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PROCESS FOR SCREENING SUBSTANCES CAPABLE OF MODULATING A RECEPTOR-DEPENDENT CELLULAR SIGNAL TRANSMISSION PATH

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(57) Claim

1. Process for determining the modulating effect of a substance on a receptor-dependent signal transduction pathway in a human or animal cell, characterised in that the modulating effect of the substance on the activity of a phospholipase or on a mechanism which precedes or succeeds the phospholipase activation in the signal transduction pathway initiated by a receptor coupled to the signal transduction pathway, is determined by incubating mammalian cells which

a) are transformed with a recombinant DNA containing a reporter gene and a regulatory sequence which responds to the change in concentration of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) brought about by the modulation of the phospholipase activity, so that the expression of the reporter gene is modulated by a change in concentration of IP_3/DAG, and which are furthermore

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b) transformed with a recombinant DNA, containing a sequence which codes for a receptor which is coupled to the phospholipase effector system so that the cells express the receptor,

with the test substance and measuring the concentration of the reporter gene product.

2. Process according to claim 1, characterised in that the recombinant DNA defined in b) contains a sequence which codes for a human receptor.

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(54) Title: PROCESS FOR SCREENING SUBSTANCES CAPABLE OF MODULATING A RECEPTOR-DEPENDENT CELLULAR SIGNAL TRANSMISSION PATH

(54) Bezeichnung: VERFAHREN ZUM SCREENEN VON SUBSTANZEN MIT MODULIERENDER WIRKUNG AUF EINEN REZEPTORABHÄNGIGEN ZELLULÄREN SIGNALÜBERTRAGUNGSWEG

(57) Abstract

A process is disclosed for screening substances having a modulating effect on a receptor-dependent signal transmission path in mammal cells. Test cells are used transformed with a reporter gene and with a regulatory sequence functionally linked thereto sensitive to the IP_3/DAG concentration, as well as with a coding DNA for a receptor coupled to the phospholipase effector system, in particular a G protein-coupled receptor. The use of reference cells without receptor ADN and reference cells with specificity for the adenylate cyclase effector system allows substances to be identified having potential pharmacological action and specificity for a determined receptor-dependent signal transmission path.

(57) Zusammenfassung

Verfahren zum Screenen von Substanzen mit modulierender Wirkung auf einen rezeptorabhängigen Signalübertragungsweg in Säugetierzellen. Es werden Testzellen eingesetzt, die transformiert sind mit einem Reportergen und einer damit funktionell verbundenen regulatorischen Sequenz, die auf die IP_3/DAG-Konzentration anspricht, sowie mit einer DNA, kodierend für einen an das Phospholipase-Effektorsystem gekoppelten Rezeptor, insbesondere einen G-Protein-gekoppelten Rezeptor. Die Verwendung von Kontrollzellen ohne Rezeptor-DNA sowie von Kontrollzellen mit Spezifität für das Adenylatzyklase-Effektorsystem erlaubt die Identifizierung von Substanzen mit potentieller pharmakologischer Wirkung, die Spezifität für einen bestimmten rezeptorabhängigen Signalübertragungsweg aufweisen.

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Process for screening substances with a modulating effect on a receptor-dependent cellular signal transduction pathway

The present invention relates to a method of determining the modulating effect of substances on a receptor-dependent signal transduction pathway in human or animal cells.

Conventional tests for discovering pharmacologically active substances are frequently assays in which a substance is investigated to find out to what extent it is able to displace a (labelled) ligand bound to a receptor (radioligand test). Tests of this kind are only able to identify those substances which influence the binding of known ligand receptor binding sites. Thus, these tests only cover the binding of the substance but not a functional response of the cell and therefore cannot distinguish whether the binding substance has an agonistic or antagonistic activity. For radioligand tests, relatively large amounts of receptors are required and frequently receptor-containing membrane fractions are used which are isolated from animal tissue. These tissues may consist of several types of cells containing different or heterologous receptors. In spite of the great significance of such substrates the heterologous composition or - in the case of investigating drugs for their pharmacological effect in humans - the species difference between humans and animals and the resulting difference in the binding properties of ligands to the human receptor and to animal receptors may give rise to problems in interpreting the results.

Many transmembrane signal transduction systems consist

of the following membrane-bound components: a) a cell surface receptor; b) a guanine-nucleotide-binding and GTP-cleaving regulatory protein, which is known as a G-protein and which can be coupled both to the receptor and to its effector; c) a so-called "effector", e.g. an ion channel or adenylate cyclases, guanylate cyclases or phospholipases.

The so-called G-protein-coupled receptors pass on the effects of very different extracellular signals such as light, smells, (peptide) hormones, neurotransmitters, etc.; they have been identified in organisms which are evolutionally as far apart as humans and yeasts (Dohlman et al., 1991). Almost all G-protein-coupled receptors have similarities with one another in their sequence; it is assumed that all are based on a similar topological motif common to them all which consists of seven hydrophobic (possibly α-helical) sections which penetrate the lipid double layer.

Cell surface receptors recognise the appropriate ligands from a variety of extracellular stimuli. The bonding of the ligand to the receptor activates a signal cascade which begins with the activation of the heterotrimeric G-protein; the activation of the receptor over a lengthy period results in desensitisation which is caused by various modifications of the receptor. The interaction of the G-protein with the activated receptor causes the replacement of guanosindiphosphate (GDP), bound to the α-subunit, by guanosintriphosphate (GTP), dissociation of the α-GTP-complex from the β-γ-heterodimer and hydrolysis of GTP into GDP. A single receptor can activate numerous G-protein molecules, thereby intensifying the ligand binding phenomenon. The α-subunit to which the GTP is bound and the free β-γ-subunit may interact with the effectors, thereby further intensifying the signal by forming so-called "second

messengers". Lower molecular second messengers such as cAMP (cyclic AMP), triggered by activation of adenylate cyclase, cGMP (cyclic GMP), triggered by activation of guanylate cyclase, or inositol-1,4,5-triphosphate (IP_3) and diacylglycerols (DAG), triggered by activation of phospholipases, optionally with the participation of hydrolases such as phospholipase C or phospholipase D (Billah et al., 1989), in turn bring about intracellular changes. These include the selective phosphorylation of proteins by activation of protein kinases (e.g. PKC by IP_3/DAG, PKA by cAMP), influencing the regulation of the transcription of certain genes, reorganisation of the cytoskeleton and depolarisation of the membrane. (A substance having an antagonistic effect can reverse the interaction caused by a substance with an agonistic effect and the resulting change in concentration of the second messenger, either wholly or partially, or may even result in a reverse functional effect). By means of this signal transduction system, cells are able to communicate with one another and coordinate their development or the effects triggered by them. The non-activated form of the G-protein is reestablished when the GTP bound to the α-subunit of the G-protein is hydrolysed to form GDP.

The specific signal transduction pathways associated with the activation of phospholipases, the reaction product of which is a DAG, or with adenylate cyclase, are hereinafter referred to as "phospholipase signal transduction pathway" (or "phospholipase effector system"), which has as its end product a DAG, or "adenylate cyclase signal transduction pathway" (or "adenylate cyclase effector system").

In mammals, approximately a hundred different G-protein-coupled receptors have been found (some of them bind the same ligands). For example, up till now, five different

muscarinic receptor subtypes have been identified, more than eight different adrenergic receptors, at least five different serotonin receptors and four different opsin receptors. A growing group of receptors and receptor subtypes which respond to purines, bombesin, bradykinin, thrombin, histamine, dopamine, ecosinoids, vasopressin, peptide hormones such as GHRH ("growth hormone releasing hormone") and somatostatin have been cloned and characterised (Dohlman et al., 1991, Simon et al., 1991; TIPS Receptor Nomenclature Supplement, 1991; Doods and von Meel, 1991). Different forms, or subtypes, of receptors which respond to the same ligand can be distinguished from one another on the basis of the intracellular reactions which they trigger. These specific receptor subtypes may be coupled with various effector systems and may regulate different ion channels. Since a single receptor subtype (possibly in the same cell or in different cells) may be coupled to more than one effector and several receptor subtypes may activate the same effectors, complicated signal transduction networks are produced. Moreover, characterisation of the G-proteins and the effectors has shown that they are also specified by large families of genes. Numerous G-proteins, various types of adenylate cyclases and phospholipases such as phospholipase C and A2 have been identified. The G-protein-dependent ion channels may also be subdivided into different protein families. At present, it is not clear which criteria determine the specificity of the interaction of the heterogeneous population of G-proteins and effector proteins, how specific receptors are connected to a specific G-protein variant, how they form autonomous circuits, in what way these signal circuits interact with one another and how they are formed afresh during the cell differentiation.

The transcription factors thus activated (e.g. CREB

protein, AP1 protein) interact with the regulatory DNA elements CRE (CRE-element, "cAMP responsive element") or TRE (TRE = "TPA responsive element": TPA = phorbol-12-myristat-13-acetate = phorbolester), which bind CREB and AP1: many genes the transcription of which is regulated by cAMP (e.g. rat somatostatin, human-α-gonadotropin) contain in the 5'-flanking region a conserved sequence as the regulatory element. This sequence is identical or similar to the palindromic octamer TGACGTCA (Montminy et al., 1990). TRE-elements contain the very similar heptameric motif TGAGTCA, which differs from the CRE-element consensus sequence only in a single nucleotide (Deutsch et al., 1988). The TRE-motif, or very similar motifs, have been identified in numerous genes the transcription of which is activated by phorbolester (Angel et al., 1987a and b; Lee et al., 1987). Surrounding DNA sequences or protein-protein interactions with other factors determine, inter alia, the concrete regulatory phenomena at a specific gene.

Because of the complexity of the network of the signal transduction pathways there may be so-called "crosstalk" between signal transduction pathways, e.g. the adylate cyclase and the phospholipase C-signal transduction pathway. The term "crosstalk" refers to the phenomenon that the influencing of one effector system also causes influencing of the other (Sassone-Corsi et al., 1990; Houslay, 1991). The phenomenon of crosstalk is used physiologically for integrating or cross-linking signals in order to produce a redundancy of signals or to ensure communication of the various signal transduction pathways. Crosstalk may occur at various planes of the signal transduction pathway, inter alia at the plane of the G-proteins. For example, a receptor or receptor subtype may interact with more than one G-protein variant, so that both the cAMP- and also the IP_3/DAG-level may possibly be affected. A possible cause of

pathological changes in the cells is the disruption of these interactions, e.g. if a specific receptor does not interact correctly, in the physiological sense, with an effector system.

There is a need for assays which make it possible to discover drugs for the treatment of pathological conditions which are specific for a certain receptor or receptor subtype and which, furthermore, specifically influence only one particular receptor-dependent signal transduction pathway.

Assays have already been developed which make use of the effect that the modulating activity of substances on the receptor-dependent signal transduction pathway can be detected by means of the expression of genes:

An assay system described by King et al., 1990, is based on the influencing of the signal transduction pathway which is used by the G-protein-coupled pheromone receptors of Saccharomyces cerevisiae, in which the reaction to the bonding of an agonistically effective compound to a receptor transfected into the yeast cell is measured in the yeast cell by colorimetry. For this purpose a modified β-adrenoreceptor gene was co-transfected under the control of the galactose-inducible GAL1-promotor (in order to achieve high expression rates), with the mammalian G-protein-subunit $G_s\alpha$ in a yeast strain which contains a reporter gene (β-galactosidase) stably integrated in the genome under the control of a FUS1-promotor which responds to pheromone. This system offers an opportunity of screening substances the agonistic effect of which activates β-galactosidase, this activation being measurable in a simple, automated assay based on a colour change. However, this system has the disadvantage that the use of a human protein isolated from its complex system in a

yeast cell cannot lead to any direct conclusions as to the processes in the human cell. Moreover, this system in yeast cells requires the co-transfection of a suitable G-protein subunit capable of interacting with the human receptor. This system does not use the signal transduction system inherent in higher cells and functional analysis of receptor-active substances in this system therefore presents problems.

Montmayeur and Borelli, 1991, described an assay which is based on the influencing of the adenylate cyclase signal transduction pathway by activation of G-protein-coupled receptors (D_2-receptors and the β-adrenergic receptor were used). The receptors were used to transform human cells which contain as reporter gene the chloramphenicol-acetyltransferase gene (CAT) under the control of a thymidine-kinase (TK) promotor. Preceding the promotor is a synthetic oligodesoxynucleotide sequence which contains a promotor element responding to cAMP ("cAMP responsive element" CRE). By means of the CAT-activity it was possible to demonstrate that agonistically activated compounds for the β-adrenergic receptor, which is known to activate adenylate cyclase, brought about a dosage-dependent increase in CAT-activity. After co-transfection with the dopamine receptors which inhibit adenylate cyclase, this activity fell back again. This showed that the cAMP-induced expression of the reporter gene can be modulated positively and negatively in dosage-dependent manner.

This assay is restricted to measuring the expression of genes which is regulated by the cAMP-concentration; it does not allow measurement of the IP_3/DAG-regulated gene expression. Interactions between the adenylate cyclase and the phospholipase C-signal transduction pathway caused by crosstalk cannot be detected with this assay either, or can only be partially detected.

The aim of the present invention was to provide a process suitable for screening substances which, depending on the receptor, modulate a phospholipase signal transduction pathway, particularly the phospholipase C-signal transduction pathway. These include substances which bind to the ligand binding site of the receptor, substances having an allosteric activity as well as substances which act non-competitively with respect to the ligand binding site. In particular, the present invention sets out to provide a process which can be automated and is thus suitable for screening substances at high throughputs and which also makes it possible to investigate complex mixtures of substances, such as extracts from organisms, for their content of pharmacologically active substances.

The present invention thus relates to a process for determining the modulating effect of a substance on a receptor-dependent signal transduction pathway in the human or animal cell. The process is characterised in that the modulating effect on the substance on the activity of a phospholipase which yields a diacylglycerol, optionally with the participation of hydrolysis, and particularly on the phospholipase C-activity or on a mechanism which precedes or follows the phospholipase C-activation in the signal transduction pathway, preferably its modulation of the signal transduction pathway triggered by G-protein-coupled receptors, is determined by incubating mammalian cells which

a) are transformed with a recombinant DNA, containing a reporter gene and a regulatory sequence which responds to the change in concentration of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) caused by the modulation of the phospholipids, particularly phospholipase C, so that the expression of the reporter

gene is modulated by a change in concentration of IP_3/DAG, and which are furthermore

b) transformed with a recombinant DNA containing a sequence coding for a receptor which is coupled to the phospholipase-effector system in such a way that the cells express the receptor,

with the substance which is to be investigated and measuring the concentration of the reporter gene product.

The recombinant DNA which responds to changes in the IP_3/DAG concentration - or in the case of control cells, the use of which will be described hereinafter, to changes in the cAMP-concentration - is also a subject of the present invention. It is hereinafter referred to as "sensor-DNA". A reporter gene is defined by the fact that its expression product is detectable and quantifiable by measuring a signal proportional to its concentration.

Regulatory sequences contained in the sensor DNA and responding to the change in concentration of IP_3/DAG contain one or more heptameric TRE-motifs, hereinafter referred to as "TRE-elements".

Regulatory elements contained in the sensor DNA which respond to the change in concentration of cAMP contain one or more octameric CRE-motifs, hereinafter referred to as CRE-elements.

Alternatively, the sensor DNA may contain a sequence coding for a protein which is only activated directly post-transcriptionally by increasing the calcium concentration.

The recombinant DNA which contairs a sequence coding for a receptor is hereinafter referred to as "receptor DNA".

According to another aspect the present invention relates to cells which are transformed only with sensor DNA. These cells are hereinafter referred to as "pre-test cells".

Cells which are transformed with sensor DNA and receptor DNA are hereinafter referred to as test cells; these cells are also the subject of the present invention.

If substances are to be investigated with respect to their pharmacological effect for the treatment of pathological conditions in man, the receptor DNA preferably contains a sequence coding for a human receptor. (The process according to the invention is preferably used for discovering substances which are suitable for treating pathological conditions in man. It may, however, also be used for screening substances used for the treatment of animals; in this case, the corresponding animal receptors are used.)

The term "substance" for the purposes of the present invention includes both pure substances and mixtures of substances.

By "modulating" effect is meant an agonistic or antagonistic effect on a receptor-dependent signal transduction pathway.

In a preferred embodiment of the present invention the cells are transformed with a DNA coding for a G-protein-coupled receptor.

According to a preferred embodiment of the process according to the invention, in addition, control cells

which are transformed only with recombinant DNA according to step a) (TRE-pretest cells) are treated with the test substance to find out whether the expression of the reporter gene can be ascribed to a receptor-dependent modulation. If the expression of the reporter gene is due to a receptor-independent modulation of the phospholipase C-effector system or, if endogenous receptors are present, a modulation of the phospholipase C-effector system dependent on these receptors, this modulation is detected by the TRE-pretest cells.

In order to investigate the specificity of the test substance on the phospholipase C-signal transduction pathway, parallel tests are conveniently carried out with pretest cells the sensor DNA of which contains a regulatory sequence which responds to the change in the cAMP-concentration caused by the modulation of adenylate cyclase (CRE-pretest cells). If desired, CRE-test cells which are transformed with the same receptor as the TRE-test cells are additionally used as control.

CRE-test cells may be used not only as control cells but also as primary substrate cells in a screening in which substances are investigated for their receptor-dependent modulating effect on the adenylate cyclase signal transduction pathway. In this case the CRE-test cells are transformed with receptor DNA which contains the sequence coding for a receptor coupled to the adenylate cyclase effector system. CRE-pretest cells are used in such a screening as control cells. If desired, TRE-pretest cells and optionally TRE-test cells as well, transformed with the identical receptor coupled to the adenylate cyclase effector system, may be used as a further control.

Cells which do not endogenously express the receptor in

question are preferred for the production of (pre)test cells, as the endogenous expression of the receptor itself, on the basis of the change in expression of the reporter gene, emits a signal which interferes with the results of the measurement. The use of cells which endogenously express the receptor is not excluded, however, provided that it is made certain that these cells strongly over-express the exogenous receptor, so that the endogenous expression by comparison thereto is negligibly small and the measuring results are not affected. In order to discover whether a cell is fundamentally suitable as a (pre)test cell for the process according to the invention, e.g. that it does not express or only slightly expresses the special receptor for which the modulating influence on the phospholipase C-effector system by the test substance is to be determined, the procedure used may be, for example, to transform a mammalian cell with TRE-sensor-DNA and subsequently treated with a substance which is known to activate the receptor in question. If the cell does not respond to this treatment or responds only to a limited extent, it can be regarded as fundamentally suitable for use as a test cell. (For testing cells for their suitability as CRE-test cells, the cells are tested analogously, with the difference that CRE-sensor DNA is used.) Another possible way of discovering whether the cell expresses the receptor is to measure the expression directly by molecular biological methods, e.g. by PCR (polymerase chain reaction) or Northern blots.

Preferably, the starting cells for the production of (pre)test cells are selected because they endogenously express none or as few as possible of the receptor types in question in order to transform them with as many different types of receptor as possible and therefore make them suitable for the production of as many test

cells as possible.

It is preferable to use cells which exhibit strong expression of the TRE-regulated reporter gene after stimulation with substances which increase the IP_3/DAG-concentration, or pretest cells which exhibit strong expression of the CRE-regulated reporter gene after stimulation with substances which increase the cAMP-level. In order to obtain test cells in which the receptors in question efficiently couple to the phospholipase C-signal transduction pathway after the action of a drug, mammalian cells are investigated for their suitability as test cells by first transforming them with sensor DNA (pretest cells) which contain one or more TRE-elements or contain a sequence coding for a protein which is activated by calcium. The pretest cell transformed with sensor DNA is then treated on the one hand with substances which bring about or simulate an increase in the cAMP-concentration (e.g. with forskolin), and on the other hand with substances which bring about or simulate an increase in the IP_3/DAG-concentration (e.g. with TPA). If the cell emits a signal only when treated with TPA but not when treated with forskolin, it satisfies the primary requisite of responding specifically to changes in the IP_3/DAG concentration, i.e. there is no crosstalk at the expression in this cell (a corresponding procedure is used for CRE-test cells; the CRE-pretest cell responds to forskolin). Furthermore, the pretest cell is expediently investigated to find out whether a coupling will occur which is physiologically "correct" for the receptor to be introduced and whether the signal transduction pathway will thus be initiated; the prerequisite for this, inter alia, is that the cell contains the G-protein variant specific to the receptor (or a G-protein variant which can functionally replace the receptor-specific one). For an investigation of

this kind, for example, the pretest cell is transformed with the receptor in question, treated with known ligands and tested to see whether modulation of the reporter gene expression takes place.

A further requisite for the suitability of a cell for use within the scope of the present invention is the stability of the cell, both in terms of its usefulness as a pretest cell (transformed only with sensor DNA) and also as a test cell (transformed with sensor and receptor DNA). In order to test the stability of the cells (viability, stable integration of the foreign DNA into the genome), experiments are carried out over a lengthy period of time under identical conditions with the pretest cells (treatment with substances which influence the concentration of second messenger) and the test cells (treatment with receptor ligands) and the reproducibility of the results is investigated.

Examples of suitable cells are those of the cell lines CHO ("Chinese hamster ovary" cell line), COS (monkey kidney cell line), A549 (human lung cancer cell line) and JEG-3 (human choriocarcinoma cell line).

The pretest cells according to the invention are used on the one hand as a starting substrate for the preparation of test cells which contain receptor DNA and on the other hand they are used in the process according to the invention as control cells for checking whether or not a signal can be put down to a receptor-dependent modulation of the signal transduction pathway by the test substance. If the substance generates a signal in the test cell and does not generate a signal in the pretest cell used as a control, the modulation of the expression of the reporter gene detected by the signal is exclusively receptor-dependent. Even though the control cell emits a signal, the substance (also)

influences a process in the signal transduction pathway which is receptor-independent; the control measurement corresponding to this signal must be subtracted from that obtained in the test cell.

The sensor DNA is preferably located on a plasmid which can be replicated in a high copy number in a suitable host organism, preferably E. coli, and after transfection into mammalian cells and integration into the host genome, permits expression of a reporter gene under the control of regulatory elements. It is preferably a shuttle vector which contains an expression cassette for the reporter gene (sensor DNA) and a selectable marker for mammalian cells as well as at least one replication origin and a marker for the replication and selection in E. coli.

In order to produce permanent cell lines which contain the sensor DNA stably integrated in their genome, the vector contains a dominant selection marker. The use of a particular selection marker is not critical; for example, it is possible to use the gene for neomycin-phosphotransferase (neo), which imparts resistance to the antibiotic geneticin (G-418) (Southern and Berg, 1982), the DHFR-gene (dihydrofolate reductase) for DHFR-deficient cells, the gene for xanthine-guanine-phosphoribosyl transferase (gpt), which imparts resistance to mycophenolic acid (Mulligan and Berg, 1981) or the hygromycin-B-phosphotransferase gene (hph; Gritz and Davies, 1983). Examples of promotors which operate the selection marker gene are the SV40 Early Promotor, the cytomegalovirus promotor (CMV-promotor), the promotor of the thymidine-kinase gene of the Herpes simplex virus (TK-promotor), the Rous Sarcoma virus (RSV) long terminal repeat (LTR). The plasmids are preferably constructed so that individual important elements such as the reporter gene, the promotor for the

reporter gene and the regulatory sequences for the selection marker can simply be exchanged or altered, in order to be able to correspond to any different requirements resulting from the particular application, e.g. as the result of the use of a different cell line. Such measures consist, for example, in incorporating multicloning sites in front of the promotor or promotors or in front of the reporter gene in order to permit the cloning of regulatory sequences which modulate the promotor or of different reporter genes.

The guiding premise for selecting a suitable reporter gene was to provide a preferably non-radioactive, automatable assay with a high degree of sensitivity.

Within the scope of the present invention it is theoretically possible to use all the reporter genes which satisfy these conditions:

Alkaline phosphatase can be measured with high sensitivity when using a chemiluminescent substrate but it does have the disadvantage that many mammalian cells express this enzyme relatively strongly. It can therefore generally be used as a reporter gene only for those cell lines which do not express it or express it only slightly.

The expression product of the β-galactosidase and β-glucuronidase gene are able to cleave the corresponding methylumbeliferyl-galactoside or -glucuronide to form fluorescent groups. These enzyme reactions are monitored using established fluorescence assays (Wieland et al., 1985; Kricka, 1988).

The expression of chloramphenicol-acetyltransferase (CAT) can admittedly be detected with relatively high sensitivity but the assay has, inter alia, the

disadvantage that it is radioactive and is difficult to automate (Hartmann, 1991).

Preferably, within the scope of the present invention, the gene coding for Photinus pyralis luciferase (De Wet et al., 1987) is used as the reporter gene. This enzyme has the advantages that it produces a high yield of bioluminescence with its substrate luciferin, with the addition of ATP, and the bioluminescence can be measured using established, automatable methods, and this enzyme is not endogenously produced by mammalian cells. Furthermore, luciferase has a relatively short half life in vivo and is not toxic even in high concentrations (Hartmann, 1991; Brasier et al., 1989).

Measurement of the activity of the firefly luciferase by means of bioluminescence is one of the most sensitive methods of measuring an enzyme. Therefore, and in view of the absence of luciferase activity in normal mammalian cells, this enzyme is particularly suitable as a reporter gene in studies of gene regulation (Subramani and DeLuca, 1987).

One disadvantage of measuring luciferase activity is the poor stability of the light signal under reaction conditions which are ideal for achieving the maximum light yield (DeLuca et al., 1979). This means that the luciferase activity is best measured in measuring equipment in which the light produced is measured directly after the addition of the substrate solution with the components required for the luminescence reaction. Another problem in determining the luciferase activity in reporter gene studies is the lysing of the cells in order to release the enzyme, which makes a further step necessary (Brasier et al., 1989).

According to another aspect the present invention thus

relates to a reagent for measuring the activity of a luciferase expressed in cell cultures.

The reagent according to the invention makes it possible to measure the luciferase activity expressed in cell cultures directly in a single step. This reagent on the one hand lyses the cells by means of a detergent and on the other hand contains the substrates necessary for the luciferase reaction. By means of this reagent, with its content of selected substances, a particularly constant light signal is obtained enabling the luciferase activity to be measured in a period of between 2 and 20 minutes after the addition of the reagent. Furthermore, this reagent is stable for at least one week in its ready-to-use state. The reagent consists of a basic buffer which contains suitable buffer substances such as tricin, HEPES, glycylglycine, phosphate, tris, preferably tricin (Leach and Webster, 1986). The pH of this buffer is in the range between 6 and 9, preferably between 7 and 8. Furthermore, a magnesium salt, preferably magnesium sulphate-heptahydrate ($MgSO_4.7H_2O$) is added to the buffer in a concentration of between 10 and 0.1 g/l, preferably 4 g/l. The substrates required for the luciferase reaction are preferably present in the following concentrations:
adenosine triphosphate (ATP) from 0.05 to 5 g/l, preferably 0.7 g/l; luciferin from 0.001 to 0.1 g/l, preferably 0.015 g/l. The buffer may additionally contain a complex-forming agent such as ethylene dinitrilotetraacetic acid disodium salt (EDTA) in a quantity of about 0.2 g/l.

The preferred basic buffer (plus substrates for the luciferase reaction) is made up of 25 mmol/l tricin, 0.5 mmol/l EDTA, 16.3 mmol/l $MgSO_4.7H_2O$, 1.2 mmol/l ATP and 0.05 mmol/l luciferin Na salt.

In order to stabilise the luciferase, a mild organic reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol is used, on its own or in admixture with other reducing agents. A reducing agent of this kind prevents the oxidation of SH-groups present in the enzyme and consequent deactivation of the luciferase during the luminescence reaction. DTT is used in a concentration of between 0.1 and 50 g/l, preferably 1 g/l. BME is added in a concentration of between 0.1 and 50 ml/l, preferably 4 ml/l.

In order to stabilise and intensify the luminescence, sodium tripolyphosphate (NaTPP) may additionally be used, and is added in a concentration of between 0.005 and 5 g/l, preferably 0.2 g/l. Instead of sodium tripolyphosphate it is also possible to use sodium pyrophosphate.

The cells are lysed using a suitable detergent such as Triton X-100, Tween 20 or 80, NP 40, Brij or the like. Triton X-100 is used in a concentration of between 0.01 and 5%, preferably 0.1%.

Alternatively, the gene coding for the enzyme apoaequorin from the source Aequoria victoria (Tanahashi et al., 1990) may be used as the reporter gene. This enzyme has the advantage that, with its cofactor coelenterazin, after binding calcium ions, it produces high yields of bioluminescence which can be measured by established automated methods. A further advantage is that this enzyme is not endogenously expressed by mammalian cells.

When constructing the sensor-DNA the reporter gene is put under the control of constituent, preferably weak promotor elements which can be modulated by one or more preceding TRE- or CRE-regulating elements. The most

suitable sensor DNA construction is determined by transiently transforming the cell with different sensor DNA plasmid constructs, varying on the one hand the reporter gene and on the other hand the control sequences, and investigating the measurement of the reporter gene product for its sensitivity. The person skilled in the art is familiar with the control sequences suitable for expression in particular mammalian cells; the choice may be made initially from the relevant literature (e.g. Landschulz et al., 1988, Turner and Tjian, 1989), and the choice may be narrowed down or perfected by means of the above-mentioned transient transfection experiments which are easy to carry out. Examples of suitable promotors are the β-globin promotor and the TK-promotor. If desired, known natural or synthetic promotors are modified, e.g. by shortening them to the minimum sequence required for the promotor function. If desired, the regulation sequence of a gene which can be induced by cAMP or IP_3/DAG may be used, this gene containing a promotor and regulatory element (Montminy et al., 1990, Deutsch et al. 1988), e.g. the 5'-regulatory sequence of the ICAM-1 gene. If the apoaequorin gene is used as reporter gene, the gene is expediently placed under the control of a strong structural promoter.

The choice of the regulatory sequence (CRE- or TRE-element including its flanking sequences) contained in the sensor DNA is generally made empirically, starting from elements known from the literature (see for example Montminy et al., 1990, Deutsch et al., 1988) which are investigated in preliminary trials for their suitability in providing a sensitively detectable inducibility of the reporter gene in a given cell system. Examples of suitable regulatory elements including the flanking sequences thereof are the sequences of somatostatin "vasoactive intestinal

peptide", cytomegalovirus enhancer, bovine leukaemia virus long terminal repeat (BLV LTR) (CRE-elements) and ICAM-1, collagenase, parathyroid hormone (TRE-elements). If the TRE- or CRE-motifs contained in the natural sequences do not have a perfect consensus sequence they, and optionally the sequences adjacent to them, may be altered by exchanging one or more nucleotides.

The regulatory elements (TRE- or CRE-elements) and the sequences flanking them may be produced synthetically or may be of natural origin. If a natural sequence is to be used which is known to respond to cAMP and/or IP_3/DAG depending on the type of cell (Karin; 1989), it is investigated in the pretest cell as to which second messenger it responds to, by treating the cells for example with TPA and forskolin.

The sensor DNA may possibly contain one or more CRE-elements in addition to one or more TRE-elements. With a sensor DNA of this kind both the activation of one or other signal transduction pathway on its own or the parallel activation of both signal transduction pathways will be detected.

In order to intensify the modulating effect of IP_3/DAG and/or cAMP on the reporter gene expression, a construct may optionally be used containing numerous regulatory CRE- and/or TRE-sequences in tandem. Preferably the regulatory sequence contains three to twelve TRE- and/or CRE-elements. When arranging the individual elements of the construct the space of the TRE- and/or CRE-elements relative to one another is selected so as to ensure that the transcription factor binds to the CRE- or TRE-elements. The optimum spacing of the regulatory TRE- or CRE-elements to one another, which is also determined in the light of the steric arrangement, is determined empirically in preliminary tests, as may also be the

spacing from other regulatory DNA elements which affect the transcription, e.g. from the TATA-box. The TRE- or CRE-elements and/or the flanking sequences may be identical or at least partially different, the latter embodiment being preferred for the tandem construction.

As sequences flanking the CRE- or TRE-element which have been found also to affect the regulating qualities of the CRE- or TRE-elements, it is preferable to use, particularly in the immediate vicinity thereof, those sequences which naturally surround the special regulatory element (Montminy et al., 1990, Deutsch et al., 1988). The sequence or arrangement thereof is determined empirically.

The elements of the sensor DNA and the marker gene used for the selection may possibly be found on two separate plasmids, one of which contains the reporter gene construct (including the expression control sequence which contains the regulatory sequence) whilst the other contains the selection marker gene construct.

Examples of suitable selection marker gene constructs are the plasmids pRSVneo, pSV2neo, pRSVgpt, pSV2gpt, the construction of which can be found in the relevant manuals, e.g. "Cloning vectors".) If separate plasmids are used the cells are co-transfected with the two plasmids and selected on the marker. The presence of the selection marker leads one to conclude that the cell also contains the reporter gene construct, since it is known that co-transformation of two genes which are located on DNA segments not physically connected to one another frequently leads to the expression of both co-transformed genes (Winnacker, 1985).

With respect to the measuring system to be used in the test proceedings it is advisable to perfect the ratio

between the maximum change and the normal value of the measuring signal, preferably by varying the construction of the sensor DNA, e.g. by structural change to the promotor arrangement. The background signal is preferably low enough to detect induction of the reporter gene expression with a high degree of sensitivity, but at the same time high enough to make it possible to determine the detection limits with respect to the negative control.

For the reception DNA construct, basically the same considerations apply as to the sensor DNA construct, except that the receptor sequence is preferably put under the control of a strong, constituent promotor. If desired, the sequence coding for the receptor and the dominant selection marker may also be on two separate plasmids, with which the cells are co-transformed, in the case of the receptor DNA as well.

The transfection of the cells with sensor or receptor DNA is carried out by conventional methods of transfection (cf. for example Potter et al. 1984; Felgner et al., 1987), the preferred methods being electroporation, calcium phosphate precipitation and lipofection.

Generally, the cells are first transformed with sensor DNA in order to obtain pretest cells and then the pretest cell is transformed with receptor DNA to produce the test cell.

The construction of the sensor DNA is preferably perfected to achieve maximum inducibility by means of a single specific effector system of the pretest or test cell, the aim being to achieve minimum inducibility by other effector systems. The TRE-test cell thus preferably responds specifically to substances which

modulate the phospholipase C-signal transduction pathway in receptor-dependent manner.

In order to rule out receptor-independent influence by the test substance, as already explained, the corresponding TRE-pretest cell is treated in parallel with the substance. In order to make it possible to pronounce on the specificity of the substance on the phospholipase C-effector system a measurement is optionally carried out, as a negative control, in which the pretest cell is transformed with CRE-sensor DNA and the test cell derived therefrom is additionally transformed with the identical receptor being investigated.)

In order to monitor the specificity of the influence on the phospholipase C-signal transduction pathway with regard to the receptor (or receptor subtype) being investigated by the substance discovered, it is convenient to carry out further control tests in which TRE-pretest cells are transformed with other different receptors and treated with the substance. If a substance is only allowed to influence one receptor specifically, which is generally the case with regard to the specificity of a drug, the substance may only modulate this one receptor.

Suitable receptors for the transfection of TRE-pretest cells in order to obtain TRE-test cells are all those receptors which are capable of coupling with the phospholipase C-signal transduction pathway. These include: α-adrenoceptor(adrenalin)-receptors of the α_1-type, angiotensin II receptors, atrionatriuretic peptide receptor, bombesin receptors, bradykinin receptors, cholecystokinin and gastrin receptors, endothelin receptors, metabotrophic excitatory amino acid receptors, histamin receptor (H_1), serotonin receptors

($5\text{-}HT_{1C}$, $5\text{-}HT_2$), leukotriene receptors (LTB_4, LTD_4), muscarinic acetylcholine receptors (M_1, M_3, M_5), neuropeptide Y-receptors (also PYY, NPP), neurotensin receptor, PAF (platelet activating factor) receptor, prostanoid receptors (EP_{1-3}, FP, TP), P_2-purioceptor (P_2gamma), tachykinin receptors ($NK_{1,2,3}$), vasopressin and oxytocin receptors (V_{1A}, V_{1B}, OT), thrombin receptor, etc. Many of these receptors may also couple to other effectors such as adenylate cyclase. The receptors mentioned and other suitable receptors as well as the effector system to which the receptors are coupled can be found in the specialist literature; a summary can be found in the TiPS Receptor Nomenclature Supplement, 1991.

Examples of non-G-protein-coupled receptors which activate phospholipase C and can therefore be used within the scope of the present invention for the transfection of the substrate cells are members of the families of the FGF-receptors, insulin receptors, PDGF-receptors, EGF-receptors, etc. (Ullrich and Schlessinger, 1990).

Receptors which are capable of coupling to the adenylate cyclase effector system and which can be used to transfect CRE-pretest cells in order to obtain CRE-test cells can also be found in the TiPS Receptor Nomenclature Supplement, 1991. Examples include the receptors for adenosine (A1, A2), for adrenalin (β- and α_2-type), for dopamine (D1, D21 (= D_{2A}), D_{2s} (= D_{2B}), for histidine (H_2-type), for serotonin ($5\text{-}HT_{1A}$- and $5\text{-}HT_{1D}$-type, $5\text{-}HT_4$), for acetylcholine (M_2- or M_4-type) and for encephalines.

(If a receptor is to be used which has not yet been cloned or of which the cDNA is not available in corresponding vectors, the receptor DNA may be obtained,

e.g. by screening of cDNA or genomic banks, and cloned.)

If a receptor is negatively coupled to adenylate cyclase (e.g. acetylcholine receptors of M_2- or M_4-type, neuropeptide Y-receptor), i.e. activation of the receptor brings about a lowering of the cAMP-concentration, the reduction in the cAMP-concentration is appropriately measured as follows: the cells are treated with the test substance; if it is an agonistically acting substance the receptor is activated, resulting in a lowering of the cAMP-level. At the same time or possibly thereafter the cell is treated with a substance which is known to increase the cAMP-concentration. It is also possible, conversely, first to increase the cAMP-concentration and then carry out the incubation with the substances being investigated. (The increase in the cAMP-level may be carried out directly, e.g. with forskolin, or indirectly by treating the cells with a substance which has an agonistic effect on a receptor which is positively coupled to adenylate cyclase. This receptor is either an endogenous receptor or a receptor with which the cell has been co-transformed.) As a control, a parallel test is set up with identical incubation conditions in which only the increase in the cAMP-concentration is determined. The difference in the signal values corresponds to the lowering of the cAMP-concentration, which can be attributed to the activity of the substance; it is a measurement of the receptor-dependent lowering of the adenylate cyclase activity. The indirect measurement of the receptor activation with negative adenylate cyclase coupling is necessary if the cAMP-concentration naturally present in the cell is very low and therefore any reduction in this concentration cannot be detected by measuring instruments.

Receptors whose activation modulates the adenylate

cyclase effector system, in addition to the phospholipase C-signal tranduction pathway, by means of crosstalk, inter alia, can also be used within the scope of the invention. If the sensor-DNA responds only to the change in concentration of PI_3/DAG (TRE-sensor-DNA), a signal will only be generated when a transcription factor is activated which binds to the TRE-element. It is irrelevant whether the adenylate cyclase effector system is also activated in parallel and the signal or part thereof is generated by crosstalk.

After transformation of the cells with receptor DNA the positive clones are investigated for expression of the receptor, e.g. using binding assays in which known radioactively labelled agonists and antagonists are used.

The number of receptors in molecules per cell can be determined by means of Scatchard blots (Human Pharmacology, 1991).

Preferably, a clone having a receptor number which corresponds as closely as possible to the physiological receptor concentration is selected from the stable transformands which contain receptor DNA. (If the receptor number is too high, incomplete and possibly non-specific coupling may take place or, in addition to the specific coupling, non-specific coupling may occur, possibly activating other effector systems at the same time. If the receptor number is too low, the signal may possibly be too low to be picked up by the measurement.)

A receptor or receptor subtype can be transfected into two different pretest cells, one of which responds to the IP3/DAG-concentration whilst the other responds to the cAMP-concentration. (The cell used in parallel is investigated with the sensor DNA specific to the other

effector system, e.g. the adenylate cyclase effector system, to discover whether it is activated specifically by means of the cAMP-signal transduction pathway, by treating it separately with substances which increase the cAMP or IP3/DAG concentration or simulate such an increase in concentration, e.g. with forskolin and TPA. For these preliminary tests the cells may either be transformed only with sensor-DNA, in which case stable transformation is not necessary, or else co-transformed with the receptor DNA; in the latter case the medium should not contain any substances which activate the receptor. Once it has been established that the cells respond only to forskolin, the assay carried out with TRE/receptor-transformed cells is repeated under the same conditions with CRE-test cells.) By comparing the data obtained with a specific substance and a specific receptor (subtype) in the specific TRE-cells and in the specific CRE-cells and in a test cell which responds to IP3/DAG and to cAMP, it is possible to establish to what extent the signal can be attributed to crosstalk and how much it can be put down to the influencing of only one of the two effector systems.

The substances to be investigated for their potential pharmacological activity by means of the process according to the invention are natural or synthetic substances and it is possible to use both pure substances and mixtures of substances (e.g. vegetable extracts, fermentation liquors, etc.). The pure substances may be, in particular, low molecular synthetic organic compounds. The substances are conveniently applied to the cells in serial dilutions in order to detect the largest possible range of concentration. The incubation time is determined empirically, e.g. by treating the given test cells with known receptor agonists and determining the moment from which the induction of the reporter gene expression can

be measured reproducibly. The incubation time is generally finished at this moment and generally comes to at least one hour. The absolute number of test cells is not critical. The number of cells will depend particularly on the detection limits of the measuring signal and on the stage of growth the cells are at, the lower limit being defined by the technological possibility of uniformly distributing the cells over the test units. If microtitre plates with 96 wells are used, the number of cells will be, for example, about 20,000 to about 200,000 cells per test unit, but it may be lower if the measuring signal is sensitive enough and the cells are accurately distributed. The growth stage at which the cells are put in depends on the cell-type-specific properties of the starting cell; furthermore, it is determined primarily by the particular receptor (in different receptors the same effector system may be activated differently or to a different intensity, depending on the stage of growth); the stage of growth and number of cells are thus also determined empirically in preliminary trials by determining the kinetics of the reporter gene expression in pretest and test cells at different growth stages.

Within the scope of the present invention it has been possible to demonstrate that different cells transformed with TRE-sensor-DNA (TRE-pretest cells) respond to the addition of substances which are known to simulate DAG (this addition being equivalent to the cell of an increase in the DAG-level caused by phospholipase C-activation), thereby inducing the expression of the reporter gene, whilst they do not respond to cAMP-increasing substances. (The sensor DNA contained as regulatory element the 1.3 kb 5'-flanking region of the human ICAM-1 gene (intercellular adhesion molecule 1), which contains a TRE-element, or a plurality of TRE-elements of the ICAM-1 gene arranged in tandem). When

the TRE-pretest cells were transformed with a receptor which is specifically coupled to the phospholipase C-signal transduction pathway (the human 5-HT_2-receptor, the neurokinin2-receptor and the human M_3-receptor were used), the expression of the luciferase gene was measured after treatment with receptor agonists; the induction mediated by the agonists was stopped by the addition of antagonists. On the other hand, this effect could not be detected if the phospholipase C-coupled receptor were transformed in pretest cells which respond to cAMP.

Within the scope of the invention it was also shown that in CHO-cells transformed with CRE-sensor-DNA (CRE-pretest cells) the expression of the CRE-regulated reporter gene is only increased by substances which increase the concentration of cAMP. The treatment of the CRE-pretest cells with substances which bring about or simulate an increase in the IP_3/DAG-concentration did not result in the induction of luciferase expression, nor did treatment with substances which are agonists for dopamine and muscarinic acetylcholine receptors. This latter result showed that the cells do not endogenously express these receptors. When the CRE-pretest cells were transfected with a receptor positively coupled to the adenylate cyclase effector system (the dopamine D_1-receptor was used), it was possible to induce the luciferase expression with agonists for the D_1-receptor and this induction was stopped again by means of antagonists.

With the aid of the present invention, a sensitive and versatile functional method of providing substances which specifically influence a signal transduction pathway in the cell in receptor-dependent manner is provided. The substances found by means of the process according to the invention serve as guide substances for

the development of drugs for treating diseases associated with a malfunction of a signal transduction pathway and may be further investigated for their pharmacological properties thereafter, e.g. in secondary screening with primary cells and only after that by clinical trials on animals. The number of animals needed will therefore be significantly reduced by the use of the process according to the invention.

The process according to the invention also has the advantage of being capable of being automated, in that the loading of the cell culture vessels, e.g. microtitre plates with 96 wells, the loading with the test substance solutions, the incubation and washing steps and the measurement, e.g. with a luminometer when luciferase is used as the reporter gene product, are carried out by robots. The process according to the invention is thus suitable for screening programmes with a high throughput capacity, with the capability of testing, for example, 2000 substances or mixtures of substances per week.

With the aid of the process according to the invention it is possible to detect allosterically acting substances and substances which act non-competitively with regard to the ligand binding site.

A further advantage of this system is that, where there are several possible ways for a substance to intervene, for a specific multistage receptor dependent intracellular signal transduction pathway, there are greater prospects of detecting the most favourable parameters for the modulation of a specific signal transduction pathway. The versatility of the system with regard to the large number of receptors and receptor subtypes which may be used enables it to be used to discover pharmacologically active substances for

different types of indications. The system according to the invention also makes it possible, with a deliberate choice of specific receptors and receptor subtypes, to distinguish with great specificity between key mechanisms in different cell systems, e.g. the central nervous system and the peripheral system.

With the aid of the process according to the invention it is also possible to clone receptors which are pharmacologically or biochemically characterised and which are known to the ligands. The operator starts with cDNA or genomic banks from which pools are transformed into the corresponding pretest cell line. The expression of the receptor is indicated by an expression of the reporter gene after the receptor has been activated by the binding of a ligand.

Schedule of Figures:

Fig. 1: Construction of the plasmid pADneo

Fig. 2: BglII - BamHI fragment after PCR for the construction of plasmid pADneoTK

Fig. 3: Construction of the plasmid pADneoTK

Fig. 4A: Plasmid pADneoTKluci

Fig. 4B: Plasmid pADneoBGluci

Fig. 5: Construction of the plasmid pRc/RSVΔNaeI

Fig. 6: Construction of the plasmid pRc/RSVneo

Fig. 7: Construction of the plasmid pADneo2BGluci

Fig. 8: Oligonucleotide sequence containing three CRE-elements

Fig. 9: Construction of the plasmid pADneo2-6C-BGL, containing six CRE-elements

Fig. 10: Plasmid pHEBo

Fig. 11: Plasmid p290

Fig. 12: Plasmid pAHygCMV1

Fig. 13: Plasmid pAHygCMV2

Fig. 14: Plasmid pBHluc1.3

Fig. 15: Plasmid pADneo(1.3ICAM)luci, containing the 5'-regulatory sequence of the ICAM-1 gene

Fig. 16: Plasmid pADneo(3TRE)BGluci, containing three TRE-elements of the ICAM-1-gene

Fig. 17: Plasmid pAD-CMV2-$5HT_2$, containing the sequence coding for the $5HT_2$-receptor

Fig. 18: Plasmid pAD-CMV2:D1, containing the sequence coding for the dopamine-D1-receptor

Fig. 19A: Plasmid pAHyg-NK2, containing the sequence coding for the NK2-receptor

Fig. 19B: Plasmid pAHyg-5HT2, containing the sequence coding for the 5HT2-receptor

Fig. 20: Induction of TRE-sensor-DNA (pBHluc1.3) by TPA in the cell lines A549, HeLa and COS-7

Fig. 21A: Induction of TRE-sensor-DNA (pBHluc1.3) by TPA, but not by forskolin, in HeLa-cells

Fig. 21B: Induction of TRE-sensor-DNA (pBHluc1.3) by

TPA, but not by forskolin, in COS 7-cells

Fig. 22: Induction of TRE-sensor-DNA (pADneo(3TRE)BGluci) by TPA, but not by forskolin in the cell lines A549, HeLa and COS 7

Fig. 23A: Induction of TRE-sensor-DNA in which three or six TRE-elements are contained at different spacings from one another, by TPA in COS 7-cells

Fig. 23B: Induction of TRE-sensor-DNA in which three of six TRE-elements are contained at different spacings from one another, by TPA in A549-cells

Fig. 24: Induction of TRE-sensor-DNA (pADneo(3TRE)BGluci) by binding an agonistically acting substance to the muscarinic M3-receptor in COS 7-cells

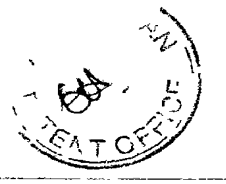
Fig. 25: A) Induction of TRE-sensor-DNA (pBHluc1.3) by binding an agonistically acting substance to the $5HT_2$-receptor in COS 7-cells, transformed with $5HT_2$-receptor DNA

B) No induction of CRE-sensor-DNA (pADneo2-C6-BGL) by a substance which acts agonistically for the $5HT_2$-receptor in COS 7-cells, transformed with $5HT_2$-receptor DNA

Fig. 26: A) Induction of TRE-sensor-DNA (pBHluc1.3) by TPA, but not by a substance which acts agonistically for the dopamine D1-receptor in COS 7-cells, transformed with dopamine D1-receptor DNA

B) Induction of CRE-sensor-DNA (pADneo2-C6-BGL) by forskolin and by binding an agonistically acting substance to the dopamine-D1-receptor in COS 7-cells, transformed with dopamine-D1-receptor-DNA

Fig. 27: Induction of luciferase expression in the TRE-pretest cell line A20 and in the TRE-test cell



line NK2-122 which expresses the human neurokinin2 receptor

Fig. 28: Kinetics of luciferase induction by means of an NK2-specific agonist

Fig. 29A: Dosage-activity curves of luciferase activity depending on the activation of the human neurokinin2-receptor by neurokinins

Fig. 29B: Dosage-activity curve of luciferase activity depending on the activation of human neurokinin2-receptor by agonists for a neurokinin receptor

Fig. 30: Inhibition of the agonistic activity of neurokinin A by NK2-specific antagonists

Fig. 31: Kinetics of the serotonin-induced luciferase expression in a test cell line which expresses the 5HT2-receptor

Fig. 32: Dosage-activity curves of luciferase activity as a function of the activation of the human 5HT2-receptor by agonists

Fig. 33: Treatment of CRE-pretest cells (CHO-cells, stably transformed with pADneo2-C6-BGL) with various substances which alter the cAMP- or IP_3/DAG-concentration or simulate a change in concentration, or with receptor-specific agonists

Fig. 34: Dosage activity curve of the transcription of activation of the luciferase gene in CRE-pretest cells by forskolin

Fig. 35A: Kinetics of luciferase induction in CHO-cells as a function of the dosage of forskolin

Fig. 35B: Kinetics of the luciferase induction in CHO-cells as a function of the dosage of forskolin (ED_{50} measurement)

Fig. 36: Treatment of CHO-pretest and test cells, stably transformed with CRE-sensor-DNA and with dopamine-D1-receptor DNA, with various substances

Figs. 37A, 37B, 38A and 38B: Dosage activity curves of luciferase activity as a function of the activation of the dopamine-D1-receptor

Figs. 39A, 39B: Dosage activity curves of luciferase activity as a function of the activation of the dopamine-D5-receptor

Fig. 40: Perfecting a reagent for measuring the luciferase activity

The invention is explained more fully by means of the Examples which follow:

The activity of the substances used in the Examples is given in the following Table:

A23187	Ca^{2+}-ionophor
Apomorphine	Agonist for the dopamine-receptor
Atropine	Antagonist for muscarinic receptors (non-specific)
Bromocryptin	Agonist for the dopamine-receptor
Carbachol	Agonist for muscarinic receptors
Clozapin	Antagonist for the dopamine-receptor (D4-specific)
dibutyryl-cAMP (dbcAMP)	membrane-permeable cAMP derivative
Dopamine	Agonist for the dopamine-receptor
Flupenthixol	Antagonist for the dopamine-receptor (D1-specific)
Forskolin	Stimulator of adenylate cyclase (increase in cAMP)
Haloperidol	Antagonist for the dopamine-receptor (D2-specific)
IBMX	Phosphodiesterase inhibitor (accumulation of cAMP)
Ketanserin	Antagonist for the serotonin-receptor ($5\text{-}HT_2$-specific)

PMA (TPA)	Protein kinase C activator, simulates IP_3/DAG
SCH23390	Antagonist for the dopamine-receptor (D1-specific)
Serotonin (5-HT)	Agonist for the serotonin-receptor
Spiroperidol (=Spiperon)	Antagonist ($5\text{-}HT_{1A}$-receptor > dopamine-receptor)
SKF-38393	Antagonist for the dopamine-receptor (D1-specific)

Example 1

Preparation of basic vectors for the expression of reporter genes in mammalian cells

a) Construction of plasmid pADneo

From parts of the plasmids pBluescript SK+ (Short et al., 1988; Stratagene, La Jolla, CA) and pRc/CMV (Invitrogen, San Diego, CA: Catalogue No. V750-20) a plasmid was prepared which contains the replication origin (ori) and selection marker for ampicillin resistance (Amp, β-lactamase) in E. coli. The intergenic region of M13 makes it possible to prepare single stranded plasmid DNA after superinfection of the transformed bacteria with a helper phage (e.g. R408 or M13K07; Stratagene) to facilitate the sequencing and mutagenesis of the plasmid DNA. Moreover, the neomycin-phosphotransferase gene (neo) under the transcriptional control of the SV40 early promotor (SV40) and the SV40 polyadenylation signal (SV40 poly(A)) are also present.

The plasmid pBluescript SK+ was linearised with HindIII and 100 ng of DNA were placed in a 100 μl PCR mixture (Saiki et al., 1988) (reaction medium: 50 mM KCl, 10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatine,

0.2 mM each of the four deoxynucleoside triphosphates (dATP, dGTP, dCTP, dTTP); 2.5 units of Taq polymerase per 100 µl). The primer used consisted of 50 pmol each of the synthetic oligonucleotides EBI-1730 (SEQ ID NO:3) (5'-GGAATTCGCGCCCTGTAGCGGCG-3') and EBI-2134 (SEQ ID NO:4) (5'-CACTGAACTCGAGCAGCTGCGTTGCTGGCGTTTTTCC-3'). After 5 minutes denaturing at 94°C PCR was carried out over 10 cycles (conditions of cycle: 40 seconds at 94°C, 45 seconds at 55°C, 5 minutes at 72°C, Perkin Elmer Cetus Thermal Cycler). The oligonucleotides flank the intergenic region of M13 or the replication origin (ori) with the intermediate gene for the β-lactamase. At the same time, at the end of the ori, an XhoI- and at the other end an EcoRI-cutting site are produced (underlined in the oligonucleotide sequence). The reaction mixture was freed from protein by extraction with phenol-chloroform and the DNA was precipitated with ethanol. The DNA obtained was cut with XhoI and EcoRI and after electrophoresis in an agarose gel a fragment of 2.2 kb was isolated.

The plasmid pRc/CMV was doubly cut with EcoRI and SalI, electrophoretically separated in an agarose gel and a 1.5 kb fragment was isolated, containing the SV40-promotor, the neo-gene and the SV40 poly(A)-signal. 100 ng of the 2.2 kb vector DNA were incubated with twice to three times the quantity of 1.5 kb insert DNA overnight at 14°C with T4 DNA ligase, then E. coli JM101 cells made competent for the uptake of DNA (Chung and Miller, 1988) were transformed and selected for ampicillin resistance. From the resulting colonies, the plasmid DNA was dissected out and characterised by cutting with various restriction enzymes. A plasmid of the desired structure was designated pADneo (Fig. 1).

b) Construction of plasmid pADneoTK

Into the plasmid pADneo was inserted the promotor region of the thymidine kinase (TK)-gene of the Herpes Simplex Virus Type I (HSV-I) flanked by two polycloning sites. This DNA fragment was produced by PCR. As a precursor for the TK-promotor, the plasmid pX1 was used (Wagner et al., 1981) and the polycloning sites were produced by lengthening the amplification primers at the 5'-end, which are no longer complementary to the precursor. 100 ng of plasmid pX1 were subjected to 20 PCR cycles (cycle conditions: 40 seconds at 94°C, 45 seconds at 55°C, 1 minute at 72°C), with 50 pmol each of the oligonucleotides EBI-2983 (SEQ ID NO:5) (5'-GACTTCAGATCTGCGGCCGCCTCGAGGGTACCGTTAACGTCGACAAACCCC-GCCCAGCGTCTTG-3') and EBI-2984 (SEQ ID NO:6) (5'-GACTTCGGATCCGAGCTCACTAGTTCTAGAAAGCTTGACGCTGTTAAGC-GGGTCGC-3'). After removal or the Taq polymerase by phenol/chloroform extraction and ethanol precipitation of the DNA, the ends were cut with BamHI and BglII (underlined sequence) and the 0.2 kb fragment was isolated after electrophoresis from an agarose gel (Fig. 2). Then this DNA was ligated with BamHI-linearised plasmid pADneo and E. coli JM101 was transformed. A resulting plasmid which contained the TK-promotor in the same orientation as the neo-gene were designated pADneoTK (Fig. 3). It contains 5' from the TK-promotor singular cutting sites for NotI, XhoI, KpnI, HpaI and SalI for the cloning of regulatory sequences which modulate the promotor, and 3' from the TK-promotor singular cutting sites for HindIII, XbaI, SpeI, SacI and BamHI for the insertion of a reporter gene.

c) Construction of plasmid pADneoTKluci

The gene for the Photinus pyralis luciferase with the SV40 poly(A) region was isolated from a derivative of

plasmid pSV232AL-AΔ5' (De Wet et al., 1987), pBHluc (Voraberger et al., 1991) as a 2.5 kb HindIII-BamHI-fragment. pADneoTK was doubly cut with HindIII and BamHI and ligated with the 2.5 kb HindIII-BamHI fragment from the plasmid pBHluc. A plasmid of the desired structure obtained after transformation of E. coli JM101 was designated pADneoTKluci (Fig. 4A). This plasmid allows the expression of luciferase under the control of the TK-promotor in mammalian cells.

d) Construction of plasmid pADneoBGluci

In order to perfect the inductibility of the reporter gene, the TK-promotor was replaced by a minimal promotor sequence of the rabbit β-globin gene. Plasmid pADneoTKluci was doubly cut with SalI and HindIII and the vector component was isolated from an agarose gel. The β-globin promotor with flanking SalI and HindIII compatible ends was prepared by the synthetic oligonucleotides EBI-3182 (SEQ ID NO:7) (5'-GACTTCGGATCCGAGCTCACTAGTTCTAGAAAGCTTGACGCTGTTAAGC-GGGTCGC-3') and EBI-3184 (SEQ ID NO:8) (5'-AGCTTGTAAGCAGCAGCTGCAGTGCTCTGCCTTTTATGCCCAAGG-3'). The two oligonucleotides were phosphorylated at the 5'-end by incubation with T4-polynucleotide kinase and ATP and subsequently ligated with the vector described above. A plasmid obtained after transformation of E. coli JM101 containing the correct sequence was designated pADneoBGluci (Fig. 4B).

e) Construction of plasmids pRc/RSVΔNaeI and pRc/RSVneo

For the use of sensor DNA in cell lines which permit the replication of plasmids with SV40 replication origin (e.g. Cos-7 (ATCC CRL1651) and 293 (ATCC CRL1573)), it was desirable for comparative studies with other cell lines to replace the SV40 promotor, which had in its

control the neogene in the plasmids described above, for another promotor (e.g. Rous Sarcoma Virus (RSV) long terminal repeat (LTR)).

In order to prepare a new expression cassette for the neo-gene, the plasmid pRc/RSVΔNaeI and pRc/RSVneo described below were prepared.

Plasmid pRc/RSV (Invitrogen, San Diego, CA; Catalogue No. V780-20) was cut with NaeI, the 3.8 kb vector component was isolated from an agarose gel and religated. As a result the 1.6 kb fragment with the neo-gene was deleted. The resulting plasmid was called pRc/RSVΔNaeI (Fig. 5).

pRc/RSVΔNaeI was doubly cut with HindIII and XbaI and the DNA ends were blunted by subsequent treatment with the Klenow fragment of the E. coli DNA polymerase (Klenow enzyme) in the presence of all four deoxynucleotides.

Plasmid pADneo was doubly cut with EcoRV and BstBI, the DNA ends were also blunted by subsequent treatment with Klenow enzyme and a 0.86 kb DNA fragment containing the neo-gene was isolated. After ligation with the vector described above and transformation, a resulting plasmid of the desired structure was called pRc/RSVneo (Fig. 6). This plasmid contains the neo-gene under the transcriptional control of the RSV promotor and the polyadenylation signal of the bovine growth hormone (bGH) and of SV40.

f) Construction of plasmid pADneo2BGluci

The expression cassette SV40 promotor - neo-gene - SV40 poly(A) signal of pADneoBGluci was exchanged for the expression cassette RSV promotor - neo-gene - bGH

poly(A) from plasmid pRc/RSVneo.

The plasmid pRc/RSVneo was cut was XhoI and the DNA ends were then blunted with Klenow enzyme. An XhoI-NotI-adaptor, prepared from the oligonucleotides EBI-3285 (SEQ ID NO:9) (5'-TCGATGCGGCCGCGACTTCAG-3') and EBI-3286 (SEQ ID NO:10) (5'-CTGAAGTCGCGGCCGCA-3') was ligated with the cut pRc/RSVneo DNA. In this way the XhoI-cutting site was destroyed and an NotI site (underlined) was inserted. After heat inactivation of the DNA-ligase the DNA was doubly cut with NruI and NotI and a 1.54 kb fragment was isolated from an agarose gel. This DNA fragment contains the RSV-neo-bGH-poly(A)-cassette.

Plasmid pADneoBGluci was partially cut with EcoRI, the ends were straightened with Klenow enzyme and then recut with NotI. A 4.9 kb long DNA fragment was isolated from an agarose gel and ligated with the 1.54 kb NruI - NotI - fragment described above. A plasmid of the desired structure obtained after transformation of *E. coli* was designated pADneo2BGluci (Fig. 7).

g) Construction of reporter plasmids with elements regulatable by cAMP (CRE-sensor-DNA)

In order to control any possible transverse sensitivity of the sensor-DNA for IP_3/DAG (containing TRE-elements) the expression cassette for the luciferase reporter gene was placed under the control of various CRE-elements. The choice of CRE-sequences was made in accordance with the composition of characterised CRE-elements (Montminy et al., 1990). The CRE-sequences chosen were those which on the one hand had the perfect 8 base long, palindromic consensus sequence TGACGTCA and on the other hand, if possible, had no longer groupings of GC-pairs, which show similarity for the recognition sequences for Sp1-transcription factors (CCGCCC or GGGCGG) in the

surrounding sequences. A number of CRE-sequences were used in tandem to intensify the modulating effect of cAMP. A combination of different CRE-sequences were used to avoid an unfavourable effect on cloning and stability in E. coli if totally identical sequences occur in several repetitions.

By inserting synthetic oligonucleotides into the plasmid pADneo2BGluci 5' from the β-globin promotor, two plasmids were produced having 3 successive CRE-sequences (pADneo2-C3BVC-BGL and pADneo2-C3SVC-BGL), and by combining these plasmids the plasmid pADneo2-C6-BGL with 6 CRE-sequences was prepared.

The triple CRE-sequences were produced by ligation of two pairs of oligonucleotides with DNA ends complementary with one another (Fig. 8). 20 pmol each of oligonucleotide EBI-3489 (SEQ ID NO:11) (5'-GGCAGCTGACGTCACTGTCTGGTGC-3') and EBI-3491 (SEQ ID NO:12) (5'-CTCCTTGGCTGACGTCAGTAGAGAGATCCCATGGC-3') were incubated in 15 μl of kinase buffer (70 mM Tris-HCl pH 7.6; 10 mM $MgCl_2$, 5 mM dithiotreitol, 2 mM ATP) with 15 units of polynucleotide-kinase for 1 hour at 37°C and the enzyme was inactivated by heat (5 minutes at 95°C). In the same way the 5'-end of the oligonucleotides EBI-3490 (SEQ ID NO:13) (5'-CTCTACTGACGTCAGC-CAAGGAGGTAC-3') and EBI-3494 (SEQ ID NO:14) (5'-CGTCATACTGTGACGTCTTTCAGACACCCCATTGACGTCAATGGGAG-3') were phosphorylated.

Equimolar amounts of the complementary oligonucleotides without the 5'-phosphate group were mixed with the phosphorylated oligonucleotides: EBI-3492 (SEQ ID NO:15) (5'-GGCCGCACCAGACAGTGACGTCAGCTGCCAGATCCCATGGC-3') with EBI-3489; EBI-3491 (SEQ ID NO:16) (5'-CTCCTTGGCTGACGTCAGTAGAGAGATCCCATGGC-3') with EBI-3490; EBI-3493 (SEQ ID NO:17)

(5'-TCGACTCCCATTGACGTCAATGGGGTGTCTGAAAGACGTCACAGTATGACG-GCCATGGGATCT-3') with EBI-3494 and added on by 5 minutes incubation at 56°C. 10 pmol of the now double-stranded oligonucleotide pairs EBI-3489 / EBI-3492 and EBI-3493 / EBI-3494 were incubated in 30 µl of ligation buffer (70 mM Tris-HCl pH 7.6; 10 mM $MgCl_2$, 5 mM dithiotreitol, 1 mM ATP) with 1 unit of T4 DNA-ligase overnight at 14°C and the enzyme was subsequently inactivated for 10 minutes at 70°C. The ligated pairs of oligonucleotides were phosphorylated at the 5'-end in 50 µl kinase buffer with 15 units of polynucleotide-kinase for 1 hour at 37°C.

In the same way the pairs of oligonucleotides EBI-3490 / EBI-3491 and EBI-3493 / EBI-3494 were joined together and then phosphorylated.

100 ng of plasmid pADneo2BGluci doubly cut with NotI and SalI were incubated with 0.2 pmol of ligated oligonucleotide complex consisting of EBI-3489/3492/3493/3494 in 20 µl of ligation buffer with 1 unit of T4 DNA-ligase for 4 hours at 22°C and then E. coli JM101 was transformed. Plasmids obtained from this were subjected to sequence analysis and a plasmid of the desired structure was designated pADneo2-C3BVC-BGL (Fig. 9). This plasmid contains 3 CRE-sequences derived from bovine leukaemia virus LTR (BLV), vasoactive intestinal peptide (VIP) and cytomegalovirus promotor (CMV). (The plasmid name refers to the sequences by the abbreviation "BVC".)

Analogously, the oligonucleotide complex EBI-3490/3491/3493/3494 was cloned in plasmid vector pADneo2BGluci doubly cut with KpnI and SalI and plasmid pADneo2-C3SVC-BGL was obtained (Fig. 9). This plasmid contains 3 CRE-sequences derived from somatostatin (Som), VIP and CMV. (The plasmid name refers to the

sequences by the abbreviation "SVC".)

In order to prepare a plasmid with 6 CRE-sequences the plasmid pADneo2-C3BVC-BGL was doubly cut with BamHI and SalI and the 3.8 kb vector component was isolated from an agarose gel. Plasmid pADneo2-C3SVC-BGL was doubly cut with BamHI and XhoI and the 2.7 kb insert was isolated. These two DNA fragments were ligated and E. coli was transformed. A plasmid of the desired structure thus obtained was designated pADneo2-C6-BGL (Fig. 9).

h) Preparation of basic vectors for the expression of genes in mammalian cells with hygromycin B resistance marker

Starting from the expression plasmids pAD-CMV1 and pAD-CMV2 (EP-A 393 438) and pHEBo (Sugden et al. 1985) plasmids were produced for the expression of genes or cDNAs under the transcriptional control of the CMV promotor/enhancer and the selection for hydromycin B resistance.

Plasmid pHEBo (Sugden et al. 1985; Fig. 10) which contains the bacterial hygromycin-B-phosphortransferase gene (Gritz and Davies 1983) under the transcriptional control of the HSV thymidine kinase promotor (McKnight 1980) was cut with ClaI, the DNA ends were filled with Klenow enzyme and then cut with BamHI. Into this vector was cloned a 0.76 kb BamHI-HindIII fragment (BamHI end filled with Klenow enzyme) with the CMV promotor/enhancer sequence (Stinski and Roehr 1985) and plasmid p290 (Fig. 11) was obtained.

The SpeI-EcoRV fragment of plasmid p290, which contains CMV promotor and EBV ori P, was cut out. Plasmid pAD-CMV1 was cut with BglII, the DNA ends were blunted with

Klenow enzyme and subsequently cut with SpeI. The gel-purified 1.6 kb DNA fragment, which contains CMV promotor, polycloning site, SV40 splice and poly(A) signals, was ligated with the p290 vector part described hereinbefore. The plasmid obtained was designated pAHygCMV1 (Fig. 12).

In the same way the SpeI-BglII fragment was isolated from plasmid pAD-CMV2 and ligated with the p290 SpeI-EcoRV vector part. The resulting plasmid, which contains the polycloning site in the opposite orientation to that in pAHygCMV1 was designated pAHygCMV2 (Fig. 13).

The full nucleotide sequence of the plasmid pAHygCMV1 is shown in SEQ ID NO:36.

The sections on the plasmid pAHygCMV1 (given in the numbering of the bases) correspond to the following sequences:

1 - 767	CMV promotor
768 - 785	T7 promotor
794 - 854	Polycloning site
854 - 1552	SV40 t intron and polyadenylation signals
1553 - 1736	5' non-coding region of the hamster DHFR gene
1737 - 2261	EBV ori P partial sequence
2262 - 2856	HSV thymidine kinase 3' non-coding region with polyadenylation signal
2857 - 3912	Hygromycin B phosphotransferase gene
3913 - 4161	HSV thymidine kinase promotor
4162 - 6531	pBR322 vector component
6532 - 6623	Linker sequences formed from various cloning processes, partly from plink322 (Maniatis et al. 1982)

The nucleotide sequence of the plasmid pAHygCMV2 is shown in SEQ ID NO:37. It differs from pAHygCMV1 only in the region of the polycloning site; the cytosine at position 856 in pAHygCMV2 corresponds to cytosine at position 849 in pAHygCMV1.

Example 2

Construction of reporter plasmids with elements (TRE-sensor-DNA) inducible by phorbolesters (TPA)

Cloning and deletion analysis of 1.3 kb of the 5'-flanking region of the human gene for intercellular adhesion molecule ICAM-1 have shown that this fragment
i) can be induced by TPA in the lung adenocarcinoma cell line A549 (ATCC CCL 185), but cannot be activated by forskolin, and
ii) contains a TPA response element (TRE) with the DNA sequence TGATTCA (Voraberger et al., 1991). The 1.3 kb long ICAM-1 fragment, or nucleotides which contain three TRE-elements in tandem orientation, was therefore used for the construction of vectors in which the luciferase gene can be induced by TPA.

a) Preparation of the plasmid pBHluc1.3

The plasmid pBHluc1.3 (Fig. 14) contains the 1.3 kb long regulating region of the ICAM-1 gene which precedes the luciferase gene. Its preparation has been described by Voraberger et al., 1991.

b) Preparation of the plasmid pADneo(1.3ICAM)luci (TRE-sensor-DNA)

From the plasmid pADneo2BGluci (see Example 1) first the β-globin promotor was cut out by cutting the plasmid

with the restriction enzymes SalI and HindIII, making the DNA ends of the plasmid blunt by adding all 4 dNTPs and Klenow enzyme, and finally religating the plasmid by the addition of T4-DNA-ligase. This plasmid without β-globin promotor, designated pADneo2luci, was then cut with NotI and KpnI, the 1.3 kb long ICAM-1 fragment was cut out of the plasmid Bluescript KS (see Voraberger et al., 1991) with NotI and KpnI, once again, and ligated into pADneo2luci. This plasmid, which was designated pADneo(1.3ICAM)luci (Fig. 15), contains the regulating and promotor region of ICAM-1, preceding the luciferase gene.

c) Preparation of the plasmid pADneo(3TRE)BGluci (TRE-sensor-DNA)

This plasmid was prepared by inserting synthetic oligonucleotides, coding for the restriction sites KpnI, BglII and XhoI, followed by 3 successive TRE-sequences, 5' from the ß-globin promotor of plasmid pADneo2BGluci. To do this, the oligonucleotides EBI-3677 (SEQ ID NO:18) (5'-GGCCGCAGGTACCAGATCTACTCGAGTGTAGACCGTGATTCAAGCTTAGCT-GTAGAC-3'), EBI-3671 (SEQ ID NO:19) (5'-GCTTGAATCACGGTCTACACTCGAGTAGATCTGGTACCTGC-3'), EBI-3678 (SEQ ID NO:20) (5'-TCGACTAAGCTTGAATCACGGTC-TACAGCTAAGCTTGAATCACGGTCTACAGCTAA-3') and EBI-3672 (SEQ ID NO:21) (5'-CGTGATTCAAGCTTAGCTGTAGACCGTGAT-TCAAGCTTAG-3') were phosphorylated and equimolar amounts of the complementary oligonucleotides EBI-3677 and EBI-3671, and EBI-3678 and EBI-3672 were added on to one another as described in Example 1. The vector pADneo2BGluci was cut with NotI and SalI, and equimolar amounts of cut plasmid pADneo2BGluci, the oligonucleotide pair EBI-3677/3671 and the oligonucleotide pair EBI-3672/3678 were mixed together and ligated to one another by the addition of T4-DNA-ligase. Resulting plasmids were subjected to sequence

analysis and a plasmid containing the desired three TRE-sequences was designated pADneo(3TRE)BGluci (Fig. 16).

d) Preparation of the plasmids pADneo(nTREdx)BGluci

These plasmids contain a number of n TRE-elements the spacing of which from one another amounts to x bases. The plasmids pADneo(3xTREd16)BGluci, pADneo(3xTREd21)BGluci, pADneo(3xTREd24)BGluci and pADneo(3xTREd34)BGluci were prepared using the following oligonucleotides as described in c):

EBI-3775 (SEQ ID NO:22) (5'-GGCCGCAGGTACCAGATCTAC-TCGAGTGTAGACCGTGATTCAAGCTTAGTGTAGAC-3') and the complementary oligonucleotide EBI-3671 (see above), EBI-3776 (SEQ ID NO:23) (5'-TCGACCTTGAATCACGGTCTACACT-AAGCTTGAATCACGGTCTACACTAA-3') and the complementary oligonucleotide EBI-3777 (SEQ ID NO:24) (5'-CGTGATTCAAGCTTAGTGTAGACCGTGATTCAAGG-3') for pADneo(3xTREd16)BGluci;

EBI-3771 (SEQU ID NO:25) (5'-GGCCGCAGGTACCAG-ATCTACTCGAGTGTAGACCGTGATTCAAGCTTAGCCTG-3') and complementary oligonucleotide EBI-3671 (see above), EBI-3772 (SEQ OD NO:26) (5'-TCGACTAAGCTTGAATCACGGTC-TACACCAGGCTAAGCTTGAATCACGGTCTACACCAGGCTAA-3') and complementary oligonucleotide EBI-3774 (SEQ ID NO:27) (5'-GTGTAGACCGTGATTCAAGCTTAGCCTGGTGTAGACCGTGATTCAAGCT-TAG-3') for pADneo(3xTREd21)BGluci;

EBI-3780 (SEQ ID NO:28) (5'-GGCCGCAGGTACCAG-ATCTACTCGAGTGTAGACCGTGATTCAAGCTTAGCCTGGCGGTGTAGAC-3') and complementary oligonucleotide EBI-3778 (SEQ ID NO:29) (5'-CCAGGCTAAGCTTGAATCACGGTCTACACTCGAGTAGATCTGGT-ACCTGC-3'), EBI-3779 (SEQ ID NO:30) (5'-CGTGATTCAAGCTTAGCCTGGCGGTGTAGACCGTGATTCAAGCTTAGC-

CTG-3') and complementary oligonucleotide EBI-3781 (SEQ ID NO:31)
(5'-TCGACAGGCTAAGCTTGAATCACGGTCTACACCGCCAGGCTAAGCTTGAA-TCACGGTCTACACCG-3') for pADneo(3xTREd24)BGluci;

EBI-3786 (SEQ ID NO:32) (5'-GGCCGCAGGTACCAGATC-TACTCGAGTGTAGACCGTGATTCAAGCTTAGCCTGGCCGGTTAGCG-CGGTGTAGAC-3') and complementary oligonucleotide EBI-3782 (SEQ ID NO:33) (5'-CGCGCTAACCGGCCAGGCT-AAGCTTGAATCACGGTCTACACTCGAGTAGATCTGGTACCTGC-3'), EBI-3790 (SEQ ID NO:34) (5'-TCGACAGGCTAAGCTTGAATCACGGTC-TACACCGCGCTAACCGGCCAGGCTAAGCTTGAATCACGGTCTACAC-3') and complementary oligonucleotide EBI-3791 (SEQ ID NO:35) (5'-CGTGATTCAAGCTTAGCCTGGCCGGTTAGCGCGGTGTAGACCGTGATTCA-AGCTTAGCCTG-3') for pADneo(3xTREd34)BGluci.

In order to obtain the corresponding plasmids with 6 TRE-elements the relevant plasmids with 3 TRE-elements were cut with NotI and XhoI and the corresponding oligonucleotides were ligated in once more. Because of the construction of the oligonucleotides this results in a duplication of the TRE-elements with the corresponding intervals. The resulting plasmids were designated pADneo(6xTREd16)BGluci, pADneo(6xTREd21)BGluci, pADneo(6xTREd24)BGluci and pADneo(6xTREd34)BGluci.

Example 3:

Cloning of G-protein coupled receptors and preparation expression plasmid (receptor DNA)

The cDNA of the human 5-HT_2-receptor in question was obtained by screening a cDNA bank and cloned into the expression vectors pAD-CMV1 or pAD-CMV2 (EP-A 393 438).

a) Isolation of a clone containing the sequence coding for the human 5-HT_2-receptor

By homology screening of a human hippocampus cDNA bank in the lambda ZAP vector (Stratagene 936205) with a clone containing the rat 5-HT_2-receptor sequence (Julius et al., 1990, Pritchett et al., 1988), a clone was isolated and the insert contained in plasmid pBluescript SK was sequenced. The resulting DNA sequence and the amino acid sequence derived from it are given in SEQ ID NO:1 and SEQ ID NO:2. Comparison of the amino acid sequence of the human 5-HT_2-receptor with the published amino acid sequence of the rat 5-HT_2-receptor (Julius et al., 1990) resulted in 90% agreement between the two amino acid sequences.

b) Subcloning of the 5-HT_2-receptor sequence in the expression vector pAD-CMV2

The expression vector pAD-CMV2 was cut with EcoRI, the DNA ends were made blunt by the addition of all 4 dNTPs and Klenow enzyme and then the vector was cut with BamHI. Into this vector was ligated the 5-HT_2-receptor sequence cut out from the plasmid pBluescript with SmaI and BamHI, and a clone thus obtained was designated pAD-CMV2-5HT_2 (Fig. 17).

c) Cloning of the dopamine-D1-receptor sequence into the expression vector pAD-CMV2

Plasmid pHD_1-gem, which contains a 3 kb EcoRI-SacI fragment of the human dopamine D1-receptor sequence in pGEM Blue Plasmid Vector (Promega) (Zhou et al., 1990), was doubly cut with EcoRI and BamHI and the 3 kb DNA-insert was isolated. Expression plasmid pAD-CMV2 (EP-A 393 438) was doubly cut with EcoRI and BamHI and the 3 kb D1-receptor fragment was cloned in targeted manner

so that the transcription of the dopamine D1 receptor is under the control of the cytomegalovirus (CMV) promotor/enhancer element. The plasmid obtained was designated pAD-CMV2:D1 (Fig. 18).

d) Subcloning of the NK2- or 5HT2-receptor sequence into the expression vector pAHygCMV1

The cDNA of the human NK2-receptor was cut out of the plasmid pBluescript-NK2 using the restriction enzymes SalI and NotI, this plasmid containing the NK2-receptor cDNA in the plasmid vector pBluescript SK+ (Stratagene) (Gerard et al., 1990), and the cDNA was cloned into the expression vector pAHygCMV1 (see Example 1 h)) and the resulting plasmid was designated pAHyg-NK2 (Fig. 19A).

The 5HT2-receptor sequence was cut out of the vector pAD-CMV2-5HT2 described above, as a XbaI-ClaI fragment, and cloned into the vector pAHygCMV2 (see Example 1H). The resulting construct was designated pAHyg-5HT2 (Fig. 19B).

Example 4:

Induction of TRE-sensor-DNA by TPA in three different pretest cell lines

The following cell lines were transiently transfected with TRE-sensor-DNA (pBHluc1.3): the human lung cancer cell line A549 (ATCC CCL 185), the human cervical cancer cell line HeLa (ATCC CCL 2) and the monkey kidney cell line COS-7 (ATCC CRL 1651), by mixing A549- and COS-7 cells in RPMI-1640 medium (Gibco) and HeLa cells in MEM medium with Earle's BSS (Gibco) with 10% heat inactivated foetal calves' serum (FCS) in each case and incubating the mixtures at 37°C in 5% CO_2. Approximately

$1x10^7$ cells per transfection were detached from the surface of the culture dish using trypsin and centrifuged for 5 minutes at 1200 rpm at ambient temperature (Heraeus Minifuge), washed once with 5 ml of serum-free medium, centrifuged for 5 minutes at 1200 rpm and suspended in 1 ml of serum-free medium. Then the cells were mixed with 250 µg/ml DEAE-dextrane, 5 µg plasmid DNA and 50 µg/ml chloroquin, incubated for 30 minutes at 37°C, washed once with medium containing no FCS and incubated overnight at 37°C with 10 ml of fresh serum-containing medium. Then the medium was changed and after 4 hours the cells were induced, either with 10 ng TPA/ml medium or with 20 µM forskolin. After another 18 hours the cells were washed with PBS, released from the petri dish using a rubber scrapper and centrifuged for 5 minutes at 1200 rpm at ambient temperature (Heraeus Minifuge). The cells were lysed by the addition of 100 µl lysing buffer (1% Triton X-100, 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EDTA and 1 mM DDT), the lysate was centrifuged for 5 minutes and the supernatant was transferred into a fresh test tube. The luciferase assay (De Wet et al., 1985) was carried out by adding 30 µl of the supernatant to 350 µl of assay buffer (25 mM glycylglycine pH 7.8, 5 mM ATP, 15 mM $MgSO_4$), placing the test tube in the luminometer Lumat 9501 (Berthold) and starting the reaction by injecting 300 µl of injection buffer (0.2mmM luciferin, 20 mM glycylglycine pH 7.8). The measuring time for light emission was 10 seconds.

a) Induction of pBHluc1.3 by TPA but not by forskolin

The cell lines A549, HeLa and COS-7 were transiently transfected as described above by the addition of plasmid pBHluc1.3 and induced by the addition of TPA. Cells which were only transfected but not induced were used as a negative control. After the specified

incubation time the cells were lysed and the luciferase assay was carried out. The results of the experiment are shown in Fig. 20 and indicate that plasmid pBHluc1.3 is inducible more than 10-fold in the cells tested. Voraberger et al., 1991 showed that this construct in A549 cells cannot be induced by forskolin. In order to verify this result for HeLa and COS-7 cells as well, these cells were transfected once more with pBHluc1.3 in another experiment and induced either with TPA or with forskolin, again using non-induced cells as control. As the result of these experiments it was shown that HeLa and COS-7 cells could also be induced by TPA but not by forskolin, as can be seen in Figures 21A and 21B.

b) Inductions of pADneo(3TRE)BGluci by TPA but not by forskolin

The cell lines A549, HeLa and COS-7 were transiently transfected by the addition of plasmid pADneo(3TRE)BGluci and induced by the addition of TPA or forskolin. Once again, cells which were only transfected but not induced were used as a negative control. After the specified incubation time the cells were lysed and the luciferase assay was carried out. The results of the experiments are shown in Fig. 22 and indicate that this vector, which contains only the TRE-elements of the ICAM-1 gene, can be induced in the tested cells by TPA but not by forskolin.

c) Induction of the plasmids pADneo(nTREdx)BGluci by TPA but not by forskolin

The cell lines COS-7 and A549 were transiently transfected by the addition of plasmid pADneo(3xTREd16)BGluci, pADneo(3xTREd21)BGluci, pADneo(3xTREd24)BGluci, pADneo(3xTREd34)BGluci, pADneo(6xTREd16)BGluci, pADneo(6xTREd21)BGluci,

pADneo(6xTREd24)BGluci or pADneo(6xTREd34)BGluci and induced by the addition of TPA or forskolin. Once again, cells which had been only transfected but not induced were used as negative control. After the specified incubation period the cells were lysed and the luciferase assay was carried out. The results of the experiment are shown in Figs. 23A and 23B and demonstrate that i) the induction with TPA for the constructions with 6 TRE-elements is greater than for the constructions with 3 TRE-elements (none of the constructions can be induced with forskolin), and ii) the induction with TPA for the constructions with 6 TRE-elements is higher when the spacing of the TRE-elements is smaller than when the spacing of the TRE-elements is greater.

Example 5

Receptor-mediated induction of TRE-sensor-DNA

In order to be able to demonstrate that sensor-DNA which is inducible with TPA can also be induced if the cell line expresses a receptor on the surface which is coupled via G-proteins to the phospholipase C-effector system, and a receptor-specific agonist is added at a suitable time, COS-7 cells were co-transfected with TRE-sensor-DNA and receptor-DNA. The use of COS-7 cells and a receptor-DNA which contains the SV40 replication origin made possible the autonomous replication of the receptor-DNA in high copy numbers and hence allowed high rates for the transient expression of the receptor on the cell surface. The co-transfection was carried out using the DEAE-dextrane method described in Example 4, except that 5 µg each of TRE-sensor-DNA and receptor-DNA were transfected. In this test series, after incubation overnight and changing the medium, either receptor-

specific agonists, or agonist and competitive antagonist, or TPA as positive control were added in parallel experiments. Once again non-induced cells were used as a negative control. After 18 hours incubation the cells were lysed and the luciferase assay was carried out as described in Example 4.

a) Induction of pADneo(3TRE)BGluci by binding an agonistically acting substance to the muscarinic M3-receptor

COS-7 cells were co-transfected by the addition of sensor-DNA pADneo(3TRE)BGluci and receptor-DNA pCD-M3 (Buckley et al., 1989), containing the sequence of the human muscarinic M3-receptor in the Okayama/Berg pCD expression vector (Okayama and Berg, 1983). Induction was carried out with i) 10 ng/ml TPA (Sigma P8139), ii) 1 mM carbachol (Sigma, C4382), iii) 1 mM carbachol and 10 μM atropine (Sigma A0132) and iv) 1 mM carbachol and 20 μM atropine. The results of the luciferase assay after incubation and lysis of the cells is shown in Fig. 24 and demonstrates that the expression of luciferase is induced both by TPA and by carbachol, an agonist for the muscarinic receptor. The induction mediated by the agonist is prevented by the simultaneous addition of the selective antagonist atropine.

b) Induction of pBHluc1.3, but not of pADneo2-C6-BGL, by binding an agonistically acting substance to the serotonin-5-HT_2-receptor

COS-7 cells were co-transfected by the addition of sensor-DNA pBHluc1.3 and receptor DNA pAD-CMV2-$5HT_2$, which contains the sequence of the human $5HT_2$-receptor in the expression vector pAD-CMV2 (see Example 3). Induction was carried out with i) 10 ng/ml medium TPA (Sigma P8139), ii) 10 μM α-methylserotonin-maleate (RBI

Research Biochemicals Incorporated M-110), and iii) 10 μM α-methylserotonin-maleate and 10 μM ketanserin-tartrate (RBI S-006). The results of the luciferase assay after incubation and lysing of the cells is shown in Fig. 25A and demonstrates that the expression of the luciferase is induced both by TPA and by α-methylserotonin-maleate, an agonist for the 5-HT_2-receptor. The induction mediated by the agonist is prevented by the simultaneous addition of the selective antagonist ketanserin-tartrate. In a parallel experiment, COS-7 cells were co-transfected by the addition of sensor DNA pADneo2-C6-BGL, which contains 6 CRE-elements, and receptor DNA pAD-CMV2-5HT_2. Induction was carried out with i) 20 μM forskolin (Sigma P8139), ii) 10 μM α-methylserotonin-maleate (RBI M-110), and iii) 10 μM α-methylserotonin-maleate and 10 μM ketanserin-tartrate (RBI S-006). The results of the luciferase assay after incubation and lysing of the cells are shown in Fig. 25B and demonstrate that the expression of luciferase is indeed induced by forskolin but not by the 5-HT_2-receptor-agonist α-methylserotonin-maleate. It follows, from the above results, that, depending on 5-HT_2-receptors, only regulation elements which respond to IP_3/DAG (TRE), but not regulation elements which respond to cAMP (CRE) are selectively activated.

c) Induction of pADneo2-C6-BGL but not of pBHluc1.3 by binding an agonistically acting substance to the dopamine-D1-receptor

COS-7 cells were co-transfected by the addition of sensor-DNA pBHluc1.3 and receptor DNA pAD-CMV2-D1, which contains the sequence of the human dopamine-D1-receptor in the expression vector pAD-CMV2 (see Example 3). Induction was carried out with i) 10 ng/ml TPA (Sigma P8139), ii) 20 μM forskolin (Sigma P8139), iii) 1 μM

apomorphine (RBI D-004), and iv) 1 μM apomorphine and 1 μM SCH23390 (RBI D-054). The results of the luciferase assay after incubation and lysis of the cells are shown in Fig. 26A) and demonstrates that the expression of the luciferase gene is indeed induced by TPA but not by apomorphine, an agonist for the D1-receptor. In a parallel experiment, COS-7 cells were co-transfected and induced by the addition of sensor-DNA pADneo2-C6-BGL, which contains 6 CRE-elements, and receptor-DNA pAD-CMV2-D1. The results of the luciferase assay after incubation and lysis of the cells are shown in Fig. 26B) and demonstrate that the expression of the luciferase is induced by forskolin and by apomorphine. The induction mediated by the agonist is prevented by the simultaneous addition of the selective antagonist SCH23390. It follows from the above results that the dopamine-D1-receptor only selectively activates regulating elements which respond to the adenylate cyclase signal transduction system (CRE), but not regulating elements which respond to the phospholipase C-signal transduction system (TRE).

Example 6

a) Development of recombinant A549 cell lines which express luciferase depending on the intracellular IP_3/DAG concentration (TRE-cell lines)

The TRE-sensor-DNA pBHluc1.3 had been induced in transient transfection experiments in the cell line A549 more than 10 times by the addition of TPA. (Example 4a). In order to produce a stable (pre-)test cell line for substances which influence the expression of the luciferase gene by direct or receptor-mediated modulation of the IP_3/DAG-signal transduction pathway, A549 cells were simultaneously transfected with the plasmid pBHluc1.3 and the selection plasmid pRSVneo by

electroporation as follows: after removal of the medium the cells were detached from the surface by means of a trypsin/PBS solution, suspended in medium and sedimented for 5 minutes at 250xg. The cells were washed in serum-free RPMI-1640 medium (Gibco), centrifuged again and resuspended in serum-free RPMI-1640 at a density of 1.25×10^7 cells/ml. 0.8 ml of cell suspension were combined with 20 µg of pBHluc1.3 and 2 µg of pRSVneo. Both plasmids had previously been linearised with BamHI. The transfection was carried out with the PG200 Progenetor II electroporation apparatus (Hoefer Scientific Instruments) with a single current impulse of 270 V, 1080 µF, 1000 msec. Then the cells were diluted in RPMI-1640 medium, mixed with 10% foetal calves' serum, and sown at a density of $2-5 \times 10^5$ cells per 90 mm culture dish. From the day after transfection onwards the cells were grown in selection medium (RPMI-1640, enriched with 10% dialysed foetal calves' serum, Na-penicillin G (100 units/ml), streptomycin (50 units/ml) and 800 µg/ml geneticin (G-418, Gibco-BRL).

15-20 days after transfection, individual cell clones were transferred into 96-well microtitre plates and further cultivated. G-418-resistant clones were tested for the inducibility of luciferase expression by the addition of TPA. Approximately 40,000 cells in each clone were sown, in a procedure repeated 6 times, in 200 µl per well of a light-impervious 96-well microtitre plate coated with tissue culture (Microlite™, Dynatech Laboratories) and incubated overnight at 37°C. Three mixtures were treated with 10 ng TPA/ml and incubated for a further 8 hours. Then the medium was removed and the cells were washed twice with PBS. The cells were taken up in 150 µl lysing buffer (25 mM tricine, 0.5 mM EDTA, 0.54 mM sodium tripolyphosphate, 6.5 mM DTT, 16.3 mM $MgSO_4.7H_2O$, 0.1% Triton X-100, 1.2 mM ATP, 0.05 mM luciferin; pH7.8) per batch and the luciferase

activity was measured in a 96 well luminometer (ML-1000, Dynatech). Cell clone A20 was selected for further experiments as it showed a measurable base level of luciferase activity as well as being 15-20-times inducible by TPA.

b) Development of recombinant A549 test cell lines which express luciferase as a function of the activation of the human neurokinin 2-receptor

This Example illustrates the preparation of a test cell line for the human neurokinin 2 (NK2)-receptor which is coupled to the phospholipase C-signal transduction pathway. This test cell line makes it possible, by measurement of the luciferase activity, to identify substances which modulate the intracellular IP_3/DAG concentrations, depending on the receptor.

The pretest cell line A20 was transfected, as described under a), by electroporation with the plasmid pAHyg-NK2, which had previously been linearised with BglII. From the day after transfection onwards, the cells were grown in selection medium, as used for the cell line A20 and additionally enriched with 150 µg/ml hygromycin B (Sigma). Individual clones were tested for inducibility of the luciferase activity as described under a). In this case, however, neurokinin A (1 µM, Sigma) was used instead of TPA as the inductor. Clone A20/NK2-122 showed, in repeated experiments a 5-7-fold induction of the luciferase activity after activation of the neurokinin 2-receptor.

As shown in Fig. 27, in the pretest cell line A20 and also in the NK2-test cell line A20/NK2-122, the expression of the luciferase is increased only via the IP_3/DAG-signal transduction pathway but not by increasing the intracellular cAMP concentration. Whereas in both

cell lines the luciferase activity could be induced up to a maximum of 18 times by TPA, depending on the dose, forskolin (the stimulator of adenylate cyclase) did not result in any induction of luciferase activity.

The kinetics of luciferase induction by means of the NK2-specific agonist NKA GR64349 (1 μM, Neosystem S.A.) in the test cell line A20/NK2-122 is shown in Fig. 28. The maximum luciferase activation was measured after an induction period of 7-8 hours. In cell line A20, on the other hand, the luciferase activity could not be induced by the addition of the NK2-agonist GR64349. This means that the pretest cell line A20 does not contain any endogenous NK2-receptor molecules and is thus a suitable control cell line for the NK2-test cell line.

Fig. 29A shows the dosage activity curves of luciferase activity as a function of the activation of the human neurokinin 2 receptor by neurokinins. The relative effectiveness of the neurokinins (Sigma), NKA > neuromedin K (NMK) > substance P (SP), in the cell line A20/NK2-122 agrees with data already described in the literature obtained from receptor binding studies. The cell line A20 could not be induced by any of the three tachykinins, which means that it does not contain any of the neurokinin receptors endogenously. The dosage-dependent activity of a series of agonists which are specific for one of the three neurokinin receptors are: NK1: GR73632 (Neosystem S.A.), BIIC 1230 ([β-Ala^4,Sar^9,Met(O_2)^{11}]SP(4-11)); NK2: GR64349 (Neosystem S.A.), BIIC 1219 ([β-Ala^8]NKA(4-10)); NK3: [MePhe^7]NMK (Bachem Feinchemikalien AG), is shown in Fig. 29B.

The agonistic effect of neurokinin A in the cell line A20/NK2-122 could be inhibited by the addition of NK2-specific antagonists (GR83074, GR87389, GR94800, Neosystem S.A.) (Fig. 30). For this purpose the cells

were simultaneously treated with a constant quantity of neurokinin A (50 nM) and increasing amounts of antagonist. NK1-specific antagonists (i.e. BIBO 2020 (±cis-3-(2-methoxybenzylamino)-2-benzhydrylquinulidine), P7492 (Peninsula Lab.)) or NK3-specific antagonists (H-9290, Bachem Feinchemikalien AG) on the other hand were effective at higher concentrations (P7492 > BIBO 2020 > H-9290).

c) Development of A549 cell lines which express luciferase as a function of the activation of the human 5HT2-receptor

The human 5HT2-receptor is another example of a receptor coupled to the phospholipase C-signal transduction pathway. The TRE-cell line A20 is therefore also suitable in this case as a starting cell line for establishing a test cell line in order to discover substances which modulate the activity of the 5HT2-receptor.

The plasmid pAHyg-5HT2, linearised with BglII, was for this purpose transfected into the cell line A20 by electroporation as described under a) and grown in selection medium. Hygromycin B-resistant cell clones were tested for the inducibility of the luciferase activity after the addition of 5HT2-specific agonist α-methylserotonin maleate (10 µM, Research Biochemicals Inc.), and the clone A20/5HT2-11, in which a 4-5-fold induction was measured, was selected for further experiments.

The kinetics of luciferase-induction by means of serotonin (1 µM) in the test cell line A20/5HT2-11 are shown in Fig. 31. As in Fig. 28 the luciferase activity increases continuously up to an induction time of 6 hours. An induction time of 6 to 8 hours is therefore

sufficient for testing agonistically or antagonistically acting substances. In cell line A20 the luciferase activity could not be induced by the addition of serotonin. This means that the pretest cell line A20 does not contain any endogenous 5HT2-receptor molecules and is thus a suitable control cell line for the 5HT2-test cell line.

Fig. 32A shows the dose-activity curves of luciferase activity as a function of the activation of the human 5HT2-receptor by agonists. As expected, the dosage-activity curves are selective for serotonin and the 5HT2-receptor agonist serotonin maleate, unlike the 5HT1A-receptor agonist 8OH-DPAT (Research Biochemicals Inc.) and buspirone.

The agonistic activity of serotonin in the cell line A20/5HT2-11 was able to be inhibited by the addition of the 5HT2-specific antagonists spiperone and mianserine (Research Biochemicals Inc.) (Fig. 32B). For this purpose the cells were simultaneously treated with a constant amount of serotonin (1 μM) and increasing amounts of antagonist.

Example 7

a) Development of recombinant Chinese hamster ovary (CHO) cell lines which express luciferase as a function of the intracellular cAMP-concentration (CRE-cell lines)

In order to prepare (pre)-test cells for investigating substances which influence the intracellular cAMP level directly or indirectly by interacting with receptor molecules, the Chinese hamster ovary cell line CHO-DXB11 (Urlaub and Chasin, 1980) was transfected with the sensor DNA pADneo2-C6-BGL.

The parental cell line CHO-DXB11 was calculated in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco), supplemented with 10% foetal calves' serum (Sebak), hypoxanthine (100 μM), thymidine (16 μM), sodium penicillin G (100 units/ml) and streptomycin (50 units/ml). One day before transfection the cells were placed in fresh medium.

The transfection by electroporation was carried out as follows: after removal of the medium the cells were detached from the surface by means of a trypsin/PBS solution, suspended in medium and pelleted for 5 minutes at 250x g. The cells were washed in HBS (10 mM HEPES pH 7.4, 150 mM NaCl) and pelleted by centrifuging. The cells were suspended in HBS in a density of 1 x 10^7 cells/ml. 0.8 ml of cell suspension were mixed with 20 μg of DNA of the plasmid pADneo2-C6-BGL linearised with ScaI and transferred into an electroporation dish. The transfection was carried out using the PG200 Progenetor II Electroporation apparatus (Hoefer Scientific Instruments, San Francisco, CA) with a single current impulse of 320 V, 1080 μF, 1000 msec. After the electroporation the cells were diluted in the medium mentioned above, 20,000 cells per 90 mm culture dish was sown and incubated overnight at 37°C. From the day after transfection onwards, the cells were cultivated with selection medium (RPMI 1640 medium supplemented with 10% foetal calves' serum, hypoxanthine (100 μM), thymidine (16 μM), sodium penicillin G (100 units/ml), and streptomycin (50 units/ml), 700 μg/ml of geneticin (G-418, Gibco-BRL) and thereafter inspected visually for cell growth.

7 to 10 days after transfection, individual cell clones which had formed were transferred into cell culture dishes with 24 wells and cultivation was continued. 25 isolated G-418 resistant cell clones were tested for the

inducibility of luciferase expression by activation of the adenylate cyclase.

Approximately 300,000 cells of each clone were sown four-fold in each well of a 6-well cell culture plate and incubated 24 hours at 37°C. Two batches of each were treated with 20 µM forskolin and incubated for a further 5 hours at 37°C. Then the medium was removed from all the cells and the cells were washed with PBS. The cells were lysed with 1% Triton X-100 and the luciferase activity was determined in a Berthold Lumat LB9501 luminometer (Brasier et al., 1989). Cell clone C6-13 was selected for further experiments because it had the highest base level of luciferase activity whilst at the same time a very high inducibility by forskolin.

Cells of the cell line CHO C6-13 were treated for 3 hours with various substances which alter the intracellular concentration of cAMP or IP_3/DAG or simulate a change in concentration, and also treated with receptor-specific agonists which influence the above-mentioned signal transduction mechanisms. As shown in Fig. 33, in the cell line CHO C6-13 the expression of the luciferase was increased only by cAMP-increasing substances such as forskolin (stimulator of adenylate cyclase) dibutyryl-cAMP (membrane-permeable cAMP-derivative and isobutylmethylxanthine (IBMX, phosphordiesterase-inhibitor). Phorbolester PMA, Ca^{2+}-ionophor A23187 and agonistic compounds for dopamine receptors (apomorphine, bromocryptin), muscarinic acetylcholine receptors (carbachol) and serotonin did not bring about any significant change in the luciferase activity. This means that the luciferase expression in the cell line CHO C6-13 is modulated only by the change in the cAMP-concentration but not by the change in the IP_3/DAG-concentration, and furthermore that the cells do not contain any biologically detectable cAMP stimulating

receptors of the dopaminergic, muscarinic or serotonin type.

The dosage-activity curve of the transcriptional activation of the luciferase reporter gene by forskolin-induced increase in the cAMP-concentration, as shown in Fig. 34, yields maximum induction at 20 μM forskolin. The drop in luciferase induction at even higher concentrations of forskolin would appear to be due to toxic effects of the forskolin itself or the excessively high cAMP level. Inhibition of phosphodiesterase by IBMX results in a reduced breakdown of the cAMP formed and consequently increased cAMP levels, with simultaneous activation of the adenylate cyclase. The maximum induction of the luciferase reporter gene was not significantly influenced by IBMX treatment, which means that the maximum activation of transcription is achieved at a cAMP concentration, obtained by 20 μM forskolin. The shift in the dosage-activity curve with IBMX by one order of magnitude to lower forskolin concentrations clearly demonstrates the accumulation of cAMP by inhibiting the enzyme responsible for its breakdown.

The kinetics of luciferase-induction as a function of the dosage of forskolin, shown in Fig. 35A, showed that 60 minutes after stimulation with forskolin an increased luciferase activity can be detected. Total induction of the luciferase expression is achieved after 2.5 hours and does not change up to 4 hours after forskolin stimulation. Although the absolute level of luciferase induction increases up to 2.5 hours, the ED_{50} levels remain virtually unaffected by the duration of the forskolin treatment (Fig. 35B).

b) Development of recombinant CHO-test cell lines which express luciferase as a function of the activation of the human dopamine-D1-receptor

This Example, like the following Example 8, demonstrates the preparation of (control) CRE-test cells which are also suitable for a cellular screening system for substances which modulate specific, preferably human adenylate cyclase-coupled receptors. The human dopamine D1-receptor sequence was used to produce receptor DNA.

The cell line CHO C6-13 characterised above was transfected, as described under a), by electroporation with plasmid pAD-CMV2:D1, which had previously been linearised with FspI. From the day after transfection onwards the cells were cultivated in selection medium for the selection according to dihydrofolate reductase (DHFR) (nucleotide-free medium α-MEM (Gibco)), 10% dialysed foetal calves' serum (Sebak), sodium penicillin G (100 units/ml), and streptomycin (50 units/ml), 700 µg/ml geneticin (G-418, Gibco-BRL) and thereafter visually inspected for cell growth. 24 individual isolated cell clones were grown in 6-well cell culture dishes as described above and 4 hours after treatment with 10 µM apomorphine (agonist for the dopamine receptor) they were tested for the increase in luciferase activity compared with untreated cells. Clone CHO 13D1-38 showed, in repeated experiments, the highest increase in luciferase activity after activation of the dopamine D1-receptor.

For the use of test cell lines in an automatic screening system with a high throughput rate it is advantageous to use microtitre plates having 96 wells per plate. For this purpose, 60,000 cells (CHO C6-13 or CHO 13D1-38) were sown in 200 µl medium per well in light-impermeable microtitre plates coated with tissue culture

(Microlite™, Dynatech Labroatories) and incubated overnight at 37°C. Then various chemicals were added to the cells and they were incubated for a certain length of time (usually 3 hours). After removal of the medium the cells were washed with PBS, lysed in 1% Triton X-100 and the luciferase activity was measured in a 96-well luminometer (ML-1000, Dynatech) (Fig. 36).

The dosage activity curves of the luciferase activity as a function of the activation of the human dopamine D1-receptor, shown in Figs. 37A and 37B and in Figs. 38A and 38B, were plotted in the microtitre plate format and represent for each dot on the curve the average of 4 separate measurements; the standard deviation was about 15%.

Analogously to the kinetics of luciferase-induction by means of forskolin in the pretest cell line CHO C6-13 (Example 7 a)) increased luciferase activity was measured in the dopamine D1-receptor test cell line CHO 13D1-38 one hour after stimulation of the D1-receptor with apomorphine (Fig. 37A). The maximum luciferase activation was achieved after 2.5 hours stimulation and remained constant for up to 4 hours. The comparison with the kinetics after direct activation of the adenylate cyclase using forskolin (Fig. 35A) leads one to conclude that the step which determines the speed is the transcription and translation of the luciferase reporter gene, but not the stimulation of the adenylate cyclase by the activated receptor.

The amended recording of the measurements for determining the ED_{50} values of apomorphine in Fig. 37B showed no significant dependency of the ED_{50} values on the duration of receptor stimulation with the agonist.

Fig. 38A shows that the induction of the reporter gene

by the activation of the D1-receptor with an agonist (Apomorphine) could be prevented in dosage-dependent manner with antagonists. For this purpose the test cells were mixed with the antagonist 24 hours after being sown in the microtitre plates and immediately afterwards a constant quantity of apomorphine (1 μM final concentration) was added, which as shown in Fig. 37A brings about maximum induction of the luciferase reporter gene. In order to simulate a screening process in which the test substances are preferably supplied in a uniform solvent, a final concentration of 1% DMSO was used in this experiment.

The effectiveness of the substances used in this test procedure correlates well with the data described in the literature by means of receptor binding studies.

Fig. 38B shows the same values recorded in another form (x-fold induction of the luciferase compared with identical untreated cells as control) and additionally the dosage-dependent activity of the agonist bromocryptin. The reversal of luciferase induction at the highest concentration of bromocryptin (100 μM) can be attributed to the cell toxicity of bromocryptin itself or to the low pH which was necessary to dissolve the bromocryptin.

Example 8

a) Development of recombinant Chinese hamster ovary (CHO) cell lines which express luciferase as a function of the intracellular cAMP-concentration (CRE-cell lines)

For the preparation of (pre)-test cells for testing substances which affect the intracellular cAMP level directly or indirectly by interaction with receptor molecules, the Chinese hamster ovary cell line CHO-DXB11

(Urlaub and Chasin, 1980) was transfected with sensor DNA pADneo2-C12-TKL. (This plasmid differs from the pADneo2-C6-BGL described in Example 1) in that the section containing the 6 CRE-elements is duplicated and the β-globin promotor is replaced by the TK-promotor (see Example 1 b)). In order to do this the SalI-HindIII-β-globin-promotor-fragment was replaced by a similarly cut fragment containing the TK-promotor (McKnight, 1980)).

The parental cell line CHO-DXB11 was cultivated in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco) supplemented with 10% foetal calves' serum (Sebak), hypoxanthine (100 μM), thymidine (16 μM), sodium penicillin G (100 units/ml), and streptomycin (50 units/ml). One day before transfection the cells were placed in fresh medium.

The transfection and testing of the cell clones were carried out as in Example 7 a). Cell clone C12-32 was selected for further experiments as it showed the highest base level of luciferase activity whilst at the same time having very high inducibility by forskolin.

b) Development of recombinant CHO-test cell lines which express luciferase as a function of the activation of the human dopamine D5-receptor

This Example (like the preceding Example 7) demonstrates the preparation of (control) CRE-test cells which are also suitable for a cellular screening system for substances which modulate specific, preferably human adenylate cyclase-coupled receptors. The human dopamine-D5-receptor sequence was used to prepare receptor DNA.

The cell line CHO C12-32 characterised earlier was

transfected as described under a) by electroporation with plasmid pAD-CMV1:D5 which had previously been linearised with 20 µg StuI. (The plasmid pAD-CMV1:D5 was prepared by ligating the 1.6 kb SalI-XbaI-fragment of phD5-gem, which contains the coding region of human D5-receptor gene (Grandy et al., 1991), into the human vector pAD-CMV1 which has also been cut.) From the day after transfection onwards the cells were cultivated in selection medium for the selection according to dihydrofolate reductase (DNFR) (nucleotide-free medium α-MEM (Gibco), 10% dialysed foetal calves' serum (Sebak), sodium penicillin G (100 units/ml), and streptomycin (50 units/ml), 700 µg/ml geneticin (G-418, Gibco-BRL) and thereafter visually inspected for cell growth. 24 individual isolated cell clones were grown in 6-well cell culture dishes as described above and 4 hours after treatment with 10 µM apomorphine (agonist for the dopamine receptor) they were investigated for any increase in luciferase activity compared with untreated cells. In repeated experiments, clone CHO 32D5-39 showed the highest increase in luciferase activity after activation of the dopamine D5-receptor.

Figure 39A shows that the induction of the reporter gene by the activation of the D5-receptor with an agonist (apomorphine) could be prevented by means of antagonists, in dosage-dependent manner. To do this, the test cells were mixed with the antagonist 24 hours after sewing in the microtitre plates and immediately afterwards a constant quantity of apomorphine (0.1 µM final concentration) was added, which brings about maximum induction of the luciferase reporter gene, as shown in Fig. 39A. The results of these experiments are shown in Fig. 39B.

The efficacy of the substances used in this test procedure correlates well with the data described in the

literature by means of receptor binding studies.

Example 9

Development of a reagent for measuring the luciferase activity

The effect of varying the concentration of ATP, luciferin, $MgSO_4.7H_2O$, dithiothreitol (DDT), β-mercaptoethanol (BME), sodium tripolyphosphate (NaTPP), Triton X-100 and the pH on the luciferase measuring signal obtained was determined (Table 2). The remaining components were present in the concentrations corresponding to the preferred basic buffer shown in Table 1. The measurements obtained 3 minutes after the addition of the reagent were used for comparison (given as percentage of the maximum measuring signal obtained).

Fig. 40 shows the effect of adding β-mercaptoethanol and/or sodium tripolyphosphate to the basic buffer on the luciferase measuring signal (filled-in squares: basic buffer; open squares: addition of 4 µl/ml β-mercaptoethanol; closed circles: addition of 0.2 mg/ml sodium tripolyphosphate; open circles: addition of 4 µl/ml β-mercaptoethanol plus 0.2 mg/ml sodium tripolyphosphate). A luminometer bearing the name Microlite ML1000 made by Dynatech was used for all the experiments.

Table 1

Substance	nmol/l	MW	g/l
Tricin *	25	179	4.48
EDTA	0.5	372	0.186
$MgSO_4.7H_2O$	16.3	246	4.0
ATP	1.2	605	0.726
Luciferin, sodium salt	0.05	302	0.015
DTT	6.5	154	1.0
NaTTP	0.54	368	0.2

* N-tris-(hydroxymethyl)methyl-glycine

Triton X-100: 1 ml/l. The pH is adjusted to 7.8 with 1 N NaOH.

Table 2

ATP-Conc (g/l)	3.30	1.47	0.65	0.29	0.13	0.06		
% max. signal	78	83	98	100	95	61		
Luciferin-Conc (mg/l)	15.0	10.0	6.67	4.44	2.96	1.98	1.32	0.88
% max. signal	100	95	88	75	65	58	42	31
$MgSO_4$-Conc (g/l)	4.00	2.67	1.78	1.19	0.79	0.53	0.35	0.23
% max. signal	100	100	97	77	77	64	48	20
DTT-Conc (g/l)	5.0	4.0	3.0	2.0	1.0	0.0		
% max. signal	71	89	95	97	100	91		
BME-Conc (ml/l)	10.0	6.67	4.44	2.96	1.98	1.32	0.88	0.59
% max. signal	93	94	100	86	84	68	82	80
NaTPP-Conc. (mg/l)	500	158	50.0	15.8	5.01	0.0		
% max. signal	75	100	82	64	54	49		
Triton-X100 (%)	0.30	0.20	0.13	0.09	0.06	0.04	0.03	0.02
% max. signal	82	88	92	100	99	97	87	28
pH	7.0	7.2	7.4	7.6	7.8	8.0	8.2	8.4
% max. signal	63	79	92	91	100	88	59	65

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SEQUENCE DATA

(1) TITLE OF INVENTION: Process for screening substances with a modulating effect on a receptor-dependent cellular signal transduction pathway

NUMBER OF SEQUENCES: 37

(2) INFORMATION FOR SEQ ID NO:1:

(i) CHARACTERISATION OF SEQUENCE:
- (A) LENGTH: 1630 base pairs
- (B) TYPE: Nucleic acid
- (C) STRAND FORM: Single strand
- (D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: cDNA

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:1:

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CGTATCGATA GCATGTCTGA TCAGACAGCA GCTGCGCAGG GCTCGGAATG CTACCCTAAA    60

AGGCCACTCG AAACCCCAGC CCCGGGAGAA CAGCATGTAC ACCAGCCTCA GTGTTACAGA   120

GTGTGGGTAC ATCAAGGTGA ATGGTGAGCA GAAACTATAA CCTGTTAGTC CTTCTACACC   180

TCATCTGCTA CAAGTTCTGG CTTAGAC ATG GAT ATT CTT TGT GAA GAA AAT       231
                              Met Asp Ile Leu Cys Glu Glu Asn
                                1               5

ACT TCT TTG AGC TCA ACT ACG AAC TCC CTA ATG CAA TTA AAT GAT GAC     279
Thr Ser Leu Ser Ser Thr Thr Asn Ser Leu Met Gln Leu Asn Asp Asp
     10                  15                  20

ACC AGG CTC TAC AGT AAT GAC TTT AAC TCC GGA GAA GCT AAC ACT TCT     327
Thr Arg Leu Tyr Ser Asn Asp Phe Asn Ser Gly Glu Ala Asn Thr Ser
 25                  30                  35                  40
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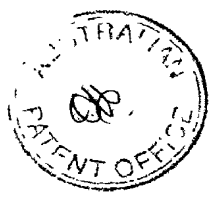

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GAT GCA TTT AAC TGG ACA GTC GAC TCT GAA AAT CGA ACC AAC CTT TCC      375
Asp Ala Phe Asn Trp Thr Val Asp Ser Glu Asn Arg Thr Asn Leu Ser
                  45                  50                  55

TGT GAA GGG TGC CTC TCA CCG TCG TGT CTC TCC TTA CTT CAT CTC CAG      423
Cys Glu Gly Cys Leu Ser Pro Ser Cys Leu Ser Leu Leu His Leu Gln
              60                  65                  70

GAA AAA AAC TGG TCT GCT TTA CTG ACA GCC GTA GTG ATT ATT CTA ACT      471
Glu Lys Asn Trp Ser Ala Leu Leu Thr Ala Val Val Ile Ile Leu Thr
          75                  80                  85

ATT GCT GGA AAC ATA CTC GTC ATC ATG GCA GTG TCC CTA GAG AAA AAG      519
Ile Ala Gly Asn Ile Leu Val Ile Met Ala Val Ser Leu Glu Lys Lys
      90                  95                  100

CTG CAG AAT GCC ACC AAC TAT TTC CTG ATG TCA CTT GCC ATA GCT GAT      567
Leu Gln Asn Ala Thr Asn Tyr Phe Leu Met Ser Leu Ala Ile Ala Asp
105                 110                 115                 120

ATG CTG CTG GGT TTC CTT GTC ATG CCC GTG TCC ATG TTA ACC ATC CTG      615
Met Leu Leu Gly Phe Leu Val Met Pro Val Ser Met Leu Thr Ile Leu
                  125                 130                 135

TAT GGG TAC CGG TGG CCT CTG CCG AGC AAG CTT TGT GCA GTC TGG ATT      663
Tyr Gly Tyr Arg Trp Pro Leu Pro Ser Lys Leu Cys Ala Val Trp Ile
              140                 145                 150

TAC CTG GAC GTG CTC TTC TCC ACG GCC TCC ATC ATG CAC CTC TGC GCC      711
Tyr Leu Asp Val Leu Phe Ser Thr Ala Ser Ile Met His Leu Cys Ala
          155                 160                 165

ATC TCG CTG GAC CGC TAC GTC GCC ATC CAG AAT CCC ATC CAC CAC AGC      759
Ile Ser Leu Asp Arg Tyr Val Ala Ile Gln Asn Pro Ile His His Ser
      170                 175                 180

CGC TTC AAC TCC AGA ACT AAG GCA TTT CTG AAA ATC ATT GCT GTT TGG      807
Arg Phe Asn Ser Arg Thr Lys Ala Phe Leu Lys Ile Ile Ala Val Trp
185                 190                 195                 200

ACC ATA TCA GTA GGT ATA TCC ATG CCA ATA CCA GTC TTT GGG CTA CAG      855
Thr Ile Ser Val Gly Ile Ser Met Pro Ile Pro Val Phe Gly Leu Gln
                  205                 210                 215
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GAC GAT TCG AAG GTC TTT AAG GAG GGG AGT TGC TTA CTC GCC GAT GAT      903
Asp Asp Ser Lys Val Phe Lys Glu Gly Ser Cys Leu Leu Ala Asp Asp
            220                 225                 230

AAC TTT GTC CTG ATC GGC TCT TTT GTG TCA TTT TTC ATT CCC TTA ACC      951
Asn Phe Val Leu Ile Gly Ser Phe Val Ser Phe Phe Ile Pro Leu Thr
        235                 240                 245

ATC ATG GTG ATC ACC TAC TTT CTA ACT ATC AAG TCA CTC CAG AAA GAA      999
Ile Met Val Ile Thr Tyr Phe Leu Thr Ile Lys Ser Leu Gln Lys Glu
    250                 255                 260

GCT ACT TTG TGT GTA AGC GAT CTT GGC ACA CGG GCC AAA TTA GCT TCT     1047
Ala Thr Leu Cys Val Ser Asp Leu Gly Thr Arg Ala Lys Leu Ala Ser
265                 270                 275                 280

TTC AGC TTC CTC CCT CAG AGT TCT TTG TCT TCA GAA AAG CTC TTC CAG     1095
Phe Ser Phe Leu Pro Gln Ser Ser Leu Ser Ser Glu Lys Leu Phe Gln
                285                 290                 295

CGG TCG ATC CAT AGG GAG CCT GGG TCC TAC ACA GGC AGG AGG ACT ATG     1143
Arg Ser Ile His Arg Glu Pro Gly Ser Tyr Thr Gly Arg Arg Thr Met
            300                 305                 310

CAG TCC ATC AGC AAT GAG CAA AAG GCA TGC AAG GTG CTG GGC ATC GTC     1191
Gln Ser Ile Ser Asn Glu Gln Lys Ala Cys Lys Val Leu Gly Ile Val
        315                 320                 325

TTC TTC CTG TTT GTG GTG ATG TGG TGC CCT TTC TTC ATC ACA AAC ATC     1239
Phe Phe Leu Phe Val Val Met Trp Cys Pro Phe Phe Ile Thr Asn Ile
    330                 335                 340

ATG GCC GTC ATC TGC AAA GAG TCC TGC AAT GAG GAT GTC ATT GGG GCC     1287
Met Ala Val Ile Cys Lys Glu Ser Cys Asn Glu Asp Val Ile Gly Ala
345                 350                 355                 360

CTG CTC AAT GTG TTT GTT TGG ATC GGT TAT CTC TCT TCA GCA GTC AAC     1335
Leu Leu Asn Val Phe Val Trp Ile Gly Tyr Leu Ser Ser Ala Val Asn
                365                 370                 375

CCA CTA GTC TAC ACA CTG TTC AAC AAG ACC TAT AGG TCA GCC TTT TCA     1383
Pro Leu Val Tyr Thr Leu Phe Asn Lys Thr Tyr Arg Ser Ala Phe Ser
            380                 385                 390

CGG TAT ATT CAG TGT CAG TAC AAG GAA AAC AAA AAA CCA TTG CAG TTA     1431
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```
Arg Tyr Ile Gln Cys Gln Tyr Lys Glu Asn Lys Lys Pro Leu Gln Leu
        395                 400                 405

ATT TTA GTG AAC ACA ATA CCG GCT TTG GCC TAC AAG TCT AGC CAA CTT     1479
Ile Leu Val Asn Thr Ile Pro Ala Leu Ala Tyr Lys Ser Ser Gln Leu
    410                 415                 420

CAA ATG GGA CAA AAA AAG AAT TCA AAG CAA GAT GCC AAG ACA ACA GAT     1527
Gln Met Gly Gln Lys Lys Asn Ser Lys Gln Asp Ala Lys Thr Thr Asp
425                 430                 435                 440

AAT GAC TGC TCA ATG GTT GCT CTA GGA AAG CAG CAT TCT GAA GAG GCT     1575
Asn Asp Cys Ser Met Val Ala Leu Gly Lys Gln His Ser Glu Glu Ala
                445                 450                 455

TCT AAA GAC AAT AGC GAC GGA GTG AAT GAA AAG GTG AGC TGT GTG TGATAGGCTA  1630
Ser Lys Asp Asn Ser Asp Gly Val Asn Glu Lys Val Ser Cys Val
            460                 465                 470
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) CHARACTERISATION OF SEQUENCE:

(A) LENGTH: 471 Amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: Protein

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:2:

```
Met Asp Ile Leu Cys Glu Glu Asn Thr Ser Leu Ser Ser Thr Thr Asn
 1               5                  10                  15

Ser Leu Met Gln Leu Asn Asp Asp Thr Arg Leu Tyr Ser Asn Asp Phe
            20                  25                  30

Asn Ser Gly Glu Ala Asn Thr Ser Asp Ala Phe Asn Trp Thr Val Asp
        35                  40                  45

Ser Glu Asn Arg Thr Asn Leu Ser Cys Glu Gly Cys Leu Ser Pro Ser
    50                  55                  60
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Cys Leu Ser Leu Leu His Leu Gln Glu Lys Asn Trp Ser Ala Leu Leu
65 70 75 80

Thr Ala Val Val Ile Ile Leu Thr Ile Ala Gly Asn Ile Leu Val Ile
85 90 95

Met Ala Val Ser Leu Glu Lys Lys Leu Gln Asn Ala Thr Asn Tyr Phe
100 105 110

Leu Met Ser Leu Ala Ile Ala Asp Met Leu Leu Gly Phe Leu Val Met
115 120 125

Pro Val Ser Met Leu Thr Ile Leu Tyr Gly Tyr Arg Trp Pro Leu Pro
130 135 140

Ser Lys Leu Cys Ala Val Trp Ile Tyr Leu Asp Val Leu Phe Ser Thr
145 150 155 160

Ala Ser Ile Met His Leu Cys Ala Ile Ser Leu Asp Arg Tyr Val Ala
165 170 175

Ile Gln Asn Pro Ile His His Ser Arg Phe Asn Ser Arg Thr Lys Ala
180 185 190

Phe Leu Lys Ile Ile Ala Val Trp Thr Ile Ser Val Gly Ile Ser Met
195 200 205

Pro Ile Pro Val Phe Gly Leu Gln Asp Asp Ser Lys Val Phe Lys Glu
210 215 220

Gly Ser Cys Leu Leu Ala Asp Asp Asn Phe Val Leu Ile Gly Ser Phe
225 230 235 240

Val Ser Phe Phe Ile Pro Leu Thr Ile Met Val Ile Thr Tyr Phe Leu
245 250 255

Thr Ile Lys Ser Leu Gln Lys Glu Ala Thr Leu Cys Val Ser Asp Leu
260 265 270

Gly Thr Arg Ala Lys Leu Ala Ser Phe Ser Phe Leu Pro Gln Ser Ser
275 280 285

Leu Ser Ser Glu Lys Leu Phe Gln Arg Ser Ile His Arg Glu Pro Gly
290 295 300

Ser Tyr Thr Gly Arg Arg Thr Met Gln Ser Ile Ser Asn Glu Gln Lys
305 310 315 320

Ala Cys Lys Val Leu Gly Ile Val Phe Phe Leu Phe Val Val Met Trp
325 330 335

Cys Pro Phe Phe Ile Thr Asn Ile Met Ala Val Ile Cys Lys Glu Ser
340 345 350

Cys Asn Glu Asp Val Ile Gly Ala Leu Leu Asn Val Phe Val Trp Ile
355 360 365

Gly Tyr Leu Ser Ser Ala Val Asn Pro Leu Val Tyr Thr Leu Phe Asn
370 375 380

Lys Thr Tyr Arg Ser Ala Phe Ser Arg Tyr Ile Gln Cys Gln Tyr Lys
385 390 395 400

Glu Asn Lys Lys Pro Leu Gln Leu Ile Leu Val Asn Thr Ile Pro Ala
405 410 415

Leu Ala Tyr Lys Ser Ser Gln Leu Gln Met Gly Gln Lys Lys Asn Ser
420 425 430

Lys Gln Asp Ala Lys Thr Thr Asp Asn Asp Cys Ser Met Val Ala Leu
435 440 445

Gly Lys Gln His Ser Glu Glu Ala Ser Lys Asp Asn Ser Asp Gly Val
450 455 460

Asn Glu Lys Val Ser Cys Val
465 470

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 23 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: Single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxyribonucleotide

(xi) SEQUENCE CHARACTERISATION: SEQ ID NO:3:

GGAATTCGCG CCCTGTAGCG GCG 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 37 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: Single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:4:

CACTGAACTC GAGCAGCTGC GTTGCTGGCG TTTTTCC 37

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 64 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:5:

GACTTCAGAT CTGCGGCCGC CTCGAGGGTA CCGTTAACGT CGACAAACCC CGCCCAGCGT 60

CTTG 64

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 56 Base pairs
(B) TYPE Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:6:

GACTTCGGAT CCGAGCTCAC TAGTTCTAGA AAGCTTGACG CTGTTAAGCG GGTCGC 56

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 56 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:7:

GACTTCGGAT CCGAGCTCAC TAGTTCTAGA AAGCTTGACG CTGTTAAGCG GGTCGC 56

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 45 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand

(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:8:

AGCTTGTAAG CAGCAGCTGC AGTGCTCTGC CTTTTATGCC CAAGG 45

(2) INFORMATION FOR SEQ ID NO:9:

(i) DESCRIPTION OF SEQUENCE:
(A) LENGTH: 21 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:9:

TCGATGCGGC CGCGACTTCA G 21

(2) INFORMATION FOR SEQ ID NO:10:

(i) DESCRIPTION OF SEQUENCE:
(A) LENGTH: 17 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonuceotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:10:

CTGAAGTCGC GGCCGCA 17

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 25 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:11:

GGCAGCTGAC GTCACTGTCT GGTGC 25

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 35 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:12:

CTCCTTGGCT GACGTCAGTA GAGAGATCCC ATGGC 35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 27 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:13:

CTCTACTGAC GTCAGCCAAG GAGGTAC 27

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 47 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:14:

CGTCATACTG TGACGTCTTT CAGACACCCC ATTGACGTCA ATGGGAG 47

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 41 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:15:

GGCCGCACCA GACAGTGACG TCAGCTGCCA GATCCCATGG C 41

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 35 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:16:

CTCCTTGGCT GACGTCAGTA GAGAGATCCC ATGGC 35

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 63 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:17:

TCGACTCCCA TTGACGTCAA TGGGGTGTCT GAAAGACGTC ACAGTATGAC GGCCATGGGA 60

TCT 63

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 57 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:18:

GGCCGCAGGT ACCAGATCTA CTCGAGTGTA GACCGTGATT CAAGCTTAGC TGTAGAC 57

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH : 41 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:19:

GCTTGAATCA CGGTCTACAC TCGAGTAGAT CTGGTACCTG C 41

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 56 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:20:

TCGACTAAGC TTGAATCACG GTCTACAGCT AAGCTTGAAT CACGGTCTAC AGCTAA 56

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 40 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:21:

CGTGATTCAA GCTTAGCTGT AGACCGTGAT TCAAGCTTAG 40

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 56 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:22:

GGCCGCAGGT ACCAGATCTA CTCGAGTGTA GACCGTGATT CAAGCTTAGT GTAGAC 56

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 50 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:23:

TCGACCTTGA ATCACGGTCT ACACTAAGCT TGAATCACGG TCTACACTAA 50

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 35 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:24:

CGTGATTCAA GCTTAGTGTA GACCGTGATT CAAGG 35

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 53 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:25:

GGCCGCAGGT ACCAGATCTA CTCGAGTGTA GACCGTGATT CAAGCTTAGC CTG 53

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 64 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:26:

TCGACTAAGC TTGAATCACG GTCTACACCA GGCTAAGCTT GAATCACGGT CTACACCAGG 60

CTAA 64

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISATION:

(A) LENGTH: 52 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:27:

GTGTAGACCG TGATTCAAGC TTAGCCTGGT CTAGACCGTG ATTCAAGCTT AG 52

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 64 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:28:

GGCCGCAGGT ACCAGATCTA CTCGAGTGTA GACCGTGATT CAAGCTTAGC CTGGCGGTGT 60

AGAC 64

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 50 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:29:

CCAGGCTAAG CTTGAATCAC GGTCTACACT CGAGTAGATC TGGTACCTGC 50

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 51 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:30:

CGTGATTCAA GCTTAGCCTG GCGGTGTAGA CCGTGATTCA AGCTTAGCCT G 51

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 65 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:31:

TCGACAGGCT AAGCTTGAAT CACGGTCTAC ACCGCCAGGC TAAGCTTGAA TCACGGTCTA 60

CACCG 65

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 74 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:32:

GGCCGCAGGT ACCAGATCTA CTCGAGTGTA GACCGTGATT CAAGCTTAGC CTGGCCGGTT 60

AGCGCGGTGT AGAC 74

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 62 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:33:

CGCGCTAACC GGCCAGGCTA AGCTTGAATC ACGGTCTACA CTCGAGTAGA TCTGGTACCT 60

GC 62

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 73 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:34:

TCGACAGGCT AAGCTTGAAT CACGGTCTAC ACCGCGCTAA CCGGCCAGGC TAAGCTTGAA 60

TCACGGTCTA CAC 73

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 61 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: synthetic oligodesoxy-ribonucleotide

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:35:

CGTGATTCAA GCTTAGCCTG GCCGGTTAGC GCGGTGTAGA CCGTGATTCA AGCTTAGCCT 60

G 61

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISATION:

(A) LENGTH: 6623 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: circular

(ii) TYPE OF MOLECULE: Plasmid-DNA

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:36:

AATCAATATT GGCAATTAGC CATATTAGTC ATTGGTTATA TAGCATGAAT CAATATTGGC 60

TATTGGCCAT TGCATACGTT GTATCTATAT CATAATATGT ACATTTATAT TGGCTCATGT 120

CCAATATGAC CGCCATGTTG ACATTGATTA TTGACTAGTT ATTAATAGTA ATCAATTACG 180

GGGTCATTAG TTCATAGCCC ATATATGGAG TTCCGCGTTA CATAACTTAC GGTAAATGGC 240

CCGCCTGGCT GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC GTATGTTCCC 300

ATAGTAACGC CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGAGTATTT ACGGTAAACT 360

GCCCACTTGG CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT TGACGTCAAT 420

GACGGTAAAT GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA CTTTCCTACT 480

TGGCAGTACA TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT TTGGCAGTAC 540

ATCAATGGGC GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA CCCCATTGAC 600

GTCAATGGGA GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG TCGTAACAAC 660

TCCGCCCCAT TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA TATAAGCAGA 720

GCTCTCTGGC TAACTAGAGA ACCCACTGCT TACTGGCTTA TCGAAATTAA TACGACTCAC 780

TATAGGGAGA CCCAAGCTTC TGCAGGTCGA CATCGATGGA TCCGGTACCT CGAGCGGCCG 840

CGAATTCTCT AGAGGATCTT TGTGAAGGAA CCTTACTTCT GTGGTGTGAC ATAATTGGAC 900

AAACTACCTA CAGAGATTTA AAGCTCTAAG GTAAATATAA AATTTTTAAG TGTATAATGT 960

GTTAAACTAC TGATTCTAAT TGTTTGTGTA TTTTAGATTC CAACCTATGG AACTGATGAA 1020

TGGGAGCAGT GGTGGAATGC CTTTAATGAG GAAAACCTGT TTTGCTCAGA AGAAATGCCA 1080

TCTAGTGATG ATGAGGCTAC TGCTGACTCT CAACATTCTA CTCCTCCAAA AAAGAAGAGA 1140

AAGGTAGAAG ACCCCAAGGA CTTTCCTTCA GAATTGCTAA GTTTTTTGAG TCATGCTGTG 1200

TTTAGTAATA GAACTCTTGC TTGCTTTGCT ATTTACACCA CAAAGGAAAA AGCTGCACTG 1260

CTATACAAGA AAATTATGGA AAAATATTTG ATGTATAGTG CCTTGACTAG AGATCATAAT 1320

CAGCCATACC ACATTTGTAG AGGTTTTACT TGCTTTAAAA AACCTCCCAC ACCTCCCCCT 1380

GAACCTGAAA CATAAAATGA ATGCAATTGT TGTTGTTAAC TTGTTTATTG CAGCTTATAA 1440

TGGTTACAAA TAAAGCAATA GCATCACAAA TTTCACAAAT AAAGCATTTT TTTCACTGCA 1500

TTCTAGTTGT GGTTTGTCCA AACTCATCAA TGTATCTTAT CATGTCTGGA TCAATTCTGA 1560

AAAACTAGCC TTAAAGACAG ACAGCTTTGT TCTAGTCAGC CAGGCAAGCA TATGTAAATA 1620

AAGTTCCTCA GGGAACTGAG GTTAAAAGAT GTATCCTGGA CCTGCCAGAC CTGGCCATTC 1680

ACGTAAACAG AAGATTCCGC CTCAAGTTCC GGTTAACAAC AGGAGGCAAC GAGATCATCG 1740

CTGTTCCTTA GGACCCTTTT ACTAACCCTA ATTCGATAGC ATATGCTTCC CGTTGGGTAA 1800

CATATGCTAT TGAATTAGGG TTAGTCTGGA TAGTATATAC TACTACCCGG GAAGCATATG 1860

CTACCCGTTT AGGGTTAACA AGGGGGCCTT ATAAACACTA TTGCTAATGC CCTCTTGAGG 1920

GTCCGCTTAT CGGTAGCTAC ACAGGCCCCT CTGATTGACG TTGGTGTAGC CTCCCGTAGT 1980

CTTCCTGGGC CCCTGGGAGG TACATGTCCC CCAGCATTGG TGTAAGAGCT TCAGCCAAGA 2040

GTTACACATA AAGGCAATGT TGTGTTGCAG TCCACAGACT GCAAAGTCTG CTCCAGGATG 2100

AAAGCCACTC AGTGTTGGCA AATGTGCACA TCCATTTATA AGGATGTCAA CTACAGTCAG 2160

AGAACCCCTT TGTGTTTGGT CCCCCCCCGT GTCACATGTG GAACAGGGCC CAGTTGGCAA 2220

GTTGTACCAA CCAACTGAAG GGATTACATG CACTGCCCCG CGTGAGCAAT ACAAAACAAA 2280

AGCGCTCCTC GTACCAGCGA AGAAGGGGCA GAGATGCCGT AGTCAGGTTT AGTTCGTCCG 2340

GCGGCGCCAG AAATCCGCGC GGTGGTTTTT GGGGGTCGGG GGTGTTTGGC AGCCACAGAC 2400

GCCCGGTGTT CGTGTCGCGC CAGTACATGC GGTCCATGCC CAGGCCATCC AAAAACCATG 2460

GGTCTGTCTG CTCAGTCCAG TCGTGGACCT GACCCCACGC AACGCCCAAA AGAATAACCC 2520

CCACGAACCA TAAACCATTC CCCATGGGGG ACCCCGTCCC TAACCCACGG GGCCCGTGGC 2580

TATGGCGGGC TTGCCGCCCC GACGTTGGCT GCGAGCCCTG GGCCTTCACC CGAACTTGGG 2640

GGTTGGGGTG GGGAAAAGGA AGAAACGCGG GCGTATTGGC CCCAATGGGG TCTCGGTGGG 2700

GTATCGACAG AGTGCCAGCC CTGGGACCGA ACCCCGCGTT TATGAACAAA CGACCCAACA 2760

CCCGTGCGTT TTATTCTGTC TTTTTATTGC CGTCATAGCG CGGGTTCCTT CCGGTATTGT 2820

CTCCTTCCGT GTTTCAGTTA GCCTCCCCCA TCTCCCGATC CCTATTCCTT TGCCCTCGGA 2880

CGAGTGCTGG GGCGTCGGTT TCCACTATCG GCGAGTACTT CTACACAGCC ATCGGTCCAG 2940

ACGGCCGCGC TTCTGCGGGC GATTTGTGTA CGCCCGACAG TCCCGGCTCC GGATCGGACG 3000

ATTGCGTCGC ATCGACCCTG CGCCCAAGCT GCATCATCGA AATTGCCGTC AACCAAGCTC 3060

TGATAGAGTT GGTCAAGACC AATGCGGAGC ATATACGCCC GGAGCCGCGG CGATCCTGCA 3120

AGCTCCGGAT GCCTCCGCTC GAAGTAGCGC GTCTGCTGCT CCATACAAGC CAACCACGGC 3180

CTCCAGAAGA AGATGTTGGC GACCTCGTAT TGGGAATCCC CGAACATCGC CTCGCTCCAG 3240

TCAATGACCG CTGTTATGCG GCCATTGTCC GTCAGGACAT TGTTGGAGCC GAAATCCGCG 3300

TGCACGAGGT GCCGGACTTC GGGGCAGTCC TCGGCCCAAA GCATCAGCTC ATCGAGAGCC 3360

TGCGCGACGG ACGCACTGAC GGTGTCGTCC ATCACAGTTT GCCAGTGATA CACATGGGGA 3420

TCAGCAATCG CGCATATGAA ATCACGCCAT GTAGTGTATT GACCGATTCC TTGCGGTCCG 3480

AATGGGCCGA ACCCGCTCGT CTGGCTAAGA TCGGCCGCAG CGATCGCATC CATGGCCTCC 3540

GCGACCGGCT GCAGAACAGC GGGCAGTTCG GTTTCAGGCA GGTCTTGCAA CGTGACACCC 3600

TGTGCACGGC GGGAGATGCA ATAGGTCAGG CTCTCGCTGA ATTCCCCAAT GTCAAGCACT 3660

TCCGGAATCG GGAGCGCGGC CGATGCAAAG TGCCGATAAA CATAACGATC TTTGTAGAAA 3720

CCATCGGCGC AGCTATTTAC CCGCAGGACA TATCCACGCC CTCCTACATC GAAGCTGAAA 3780

GCACGAGATT CTTCGCCCTC CGAGAGCTGC ATCAGGTCGG AGACGCTGTC GAACTTTTCG 3840

ATCAGAAACT TCTCGACAGA CGTCGCGGTG AGTTCAGGCT TTTTCATATC TCATTGCCCC 3900

CGGACGAGGA TCTGCGGCAC GCTGTTGACG CTGTTAAGCG GGTCGCTGCA GGGTCGCTCG 3960

GTGTTCGAGG CCACACGCGT CACCTTAATA TGCGAAGTGG ACCTGGGACC GCGCCGCCCC 4020

GACTGCATCT GCGTGTTCGA ATTCGCCAAT GACAAGACGC TGGGCGGGGT TTGTGTCATC 4080

ATAGAACTAA AGACATGCAA ATATATTTCT TCCGGGGACA CCGCCAGCAA ACGCGAGCAA 4140

CGGGCCACGG GGATGAAGCA GGGCGGCACC TCGCTAACGG ATTCACCACT CCAAGAATTG 4200

GAGCCAATCA ATTCTTGCGG AGAACTGTGA ATGCGCAAAC CAACCCTTGG CAGAACATAT 4260

CCATCGCGTC CGCCATCTCC AGCAGCCGCA CGCGGCGCAT CTCGGGCAGC GTTGGGTCCT 4320

GGCCACGGGT GCGCATGATC GTGCTCCTGT CGTTGAGGAC CCGGCTAGGC TGGCGGGGTT 4380

GCCTTACTGG TTAGCAGAAT GAATCACCGA TACGCGAGCG AACGTGAAGC GACTGCTGCT 4440

GCAAAACGTC TGCGACCTGA GCAACAACAT GAATGGTCTT CGGTTTCCGT GTTTCGTAAA 4500

GTCTGGAAAC GCGGAAGTCA GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG 4560

GTCGTTCGGC TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA 4620

GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC 4680

CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC 4740

AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG 4800

TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC 4860

CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT 4920

CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG 4980

CCCGACCGCT GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC 5040

TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT 5100

GCTACAGAGT TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT 5160

ATCTGCGCTC TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC 5220

AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA 5280

AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC 5340

GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC 5400

CTTTTAAATT AAAAATGAAG TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT 5460

GACAGTTACC AATGCTTAAT CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCGTTCA 5520

TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG CTTACCATCT 5580

GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA 5640

ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC 5700

ATCCAGTCTA TTAATTGTTG CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG 5760

CGCAACGTTG TTGCCATTGC TGCAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT 5820

TCATTCAGCT CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA 5880

AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA GTAAGTTGGC CGCAGTGTTA 5940

TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC 6000

TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG 6060

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GTGCTCATCA TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG 6180

AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC 6240

ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG 6300

GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT 6360

CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA 6420

GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC 6480

ATGACATTAA CCTATAAAAA TAGGCGTATC ACGAGGCCCT TTCGTCTTCA AGAATTCTCA 6540

TGTTTGACAG CTTATCGATC CGGCCAACGG TGTTGCCATT GCTGCAGGCG CAGAGCTGGT 6600

AGGTATGGAA GATCTATACA GTG 6623

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISATION:
(A) LENGTH: 6630 Base pairs
(B) TYPE: Nucleic acid
(C) STRAND FORM: single strand
(D) TOPOLOGY: circular

(ii) TYPE OF MOLECULE: Plasmid-DNA

(xi) DESCRIPTION OF SEQUENCE: SEQ ID NO:37:

AATCAATATT GGCAATTAGC CATATTAGTC ATTGGTTATA TAGCATGAAT CAATATTGGC 60

TATTGGCCAT TGCATACGTT GTATCTATAT CATAATATGT ACATTTATAT TGGCTCATGT 120

CCAATATGAC CGCCATGTTG ACATTGATTA TTGACTAGTT ATTAATAGTA ATCAATTACG 180

GGGTCATTAG TTCATAGCCC ATATATGGAG TTCCGCGTTA CATAACTTAC GGTAAATGGC 240

CCGCCTGGCT GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC GTATGTTCCC 300

ATAGTAACGC CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGAGTATTT ACGGTAAACT 360

GCCCACTTGG CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT TGACGTCAAT 420

GACGGTAAAT GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA CTTTCCTACT 480

TGGCAGTACA TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT TTGGCAGTAC 540

ATCAATGGGC GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA CCCCATTGAC 600

GTCAATGGGA GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG TCGTAACAAC 660

TCCGCCCCAT TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA TATAAGCAGA 720

GCTCTCTGGC TAACTAGAGA ACCCACTGCT TACTGGCTTA TCGAAATTAA TACGACTCAC 780

TATAGGGAGA CCCAAGCTCT AGAGAATTCG CGGCTCGAGG TACCGGATCC ATCGATGTCG 840

ACCTGCAGAA GCTTGCTAGA GGATCTTTGT GAAGGAACCT TACTTCTGTG GTGTGACATA 900

ATTGGACAAA CTACCTACAG AGATTTAAAG CTCTAAGGTA AATATAAAAT TTTTAAGTGT 960

ATAATGTGTT AAACTACTGA TTCTAATTGT TTGTGTATTT TAGATTCCAA CCTATGGAAC 1020

TGATGAATGG GAGCAGTGGT GGAATGCCTT TAATGAGGAA AACCTGTTTT GCTCAGAAGA 1080

AATGCCATCT AGTGATGATG AGGCTACTGC TGACTCTCAA CATTCTACTC CTCCAAAAAA 1140

GAAGAGAAAG GTAGAAGACC CCAAGGACTT TCCTTCAGAA TTGCTAAGTT TTTTGAGTCA 1200

TGCTGTGTTT AGTAATAGAA CTCTTGCTTG CTTTGCTATT TACACCACAA AGGAAAAAGC 1260

TGCACTGCTA TACAAGAAAA TTATGGAAAA ATATTTGATG TATAGTGCCT TGACTAGAGA 1320

TCATAATCAG CCATACCACA TTTGTAGAGG TTTTACTTGC TTTAAAAAAC CTCCCACACC 1380

TCCCCCTGAA CCTGAAACAT AAAATGAATG CAATTGTTGT TGTTAACTTG TTTATTGCAG 1440

CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA GCATTTTTTT 1500

CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGATCA 1560

ATTCTGAGAA ACTAGCCTTA AAGACAGACA GCTTTGTTCT AGTCAGCCAG GCAAGCATAT 1620

GTAAATAAAG TTCCTCAGGG AACTGAGGTT AAAAGATGTA TCCTGGACCT GCCAGACCTG 1680

GCCATTCACG TAAACAGAAG ATTCCGCCTC AAGTTCCGGT TAACAACAGG AGGCAACGAG 1740

ATCATCGCTG TTCCTTAGGA CCCTTTTACT AACCCTA'TT CGATAGCATA TGCTTCCCGT 1800

TGGGTAACAT ATGCTATTGA ATTAGGGTTA GTCTGGATAG TATATACTAC TACCCGGGAA 1860

GCATATGCTA CCCGTTTAGG GTTAACAAGG GGGCCTTATA AACACTATTG CTAATGCCCT 1920

CTTGAGGGTC CGCTTATCGG TAGCTACACA GGCCCCTCTG ATTGACGTTG GTGTAGCCTC 1980

CCGTAGTCTT CCTGGGCCCC TGGGAGGTAC ATGTCCCCCA GCATTGGTGT AAGAGCTTCA 2040

GCCAAGAGTT ACACATAAAG GCAATGTTGT GTTGCAGTCC ACAGACTGCA AAGTCTGCTC 2100

CAGGATGAAA GCCACTCAGT GTTGGCAAAT GTGCACATCC ATTTATAAGG ATGTCAACTA 2160

CAGTCAGAGA ACCCCTTTGT GTTTGGTCCC CCCCCGTGTC ACATGTGGAA CAGGGCCCAG 2220

TTGGCAAGTT GTACCAACCA ACTGAAGGGA TTACATGCAC TGCCCCGCGT GAGCAATACA 2280

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CACAGACGCC CGGTGTTCGT GTCGCGCCAG TACATGCGGT CCATGCCCAG GCCATCCAAA 2460

AACCATGGGT CTGTCTGCTC AGTCCAGTCG TGGACCTGAC CCCACGCAAC GCCCAAAAGA 2520

ATAACCCCCA CGAACCATAA ACCATTCCCC ATGGGGGACC CCGTCCCTAA CCCACGGGGC 2580

CCGTGGCTAT GGCGGGCTTG CCGCCCCGAC GTTGGCTGCG AGCCCTGGGC CTTCACCCGA 2640

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CCCAACACCC GTGCGTTTTA TTCTGTCTTT TTATTGCCGT CATAGCGCGG GTTCCTTCCG 2820

GTATTGTCTC CTTCCGTGTT TCAGTTAGCC TCCCCCATCT CCCGATCCCT ATTCCTTTGC 2880

CCTCGGACGA GTGCTGGGGC GTCGGTTTCC ACTATCGGCG AGTACTTCTA CACAGCCATC 2940

GGTCCAGACG GCCGCGCTTC TGCGGGCGAT TTGTGTACGC CCGACAGTCC CGGCTCCGGA 3000

TCGGACGATT GCGTCGCATC GACCCTGCGC CCAAGCTGCA TCATCGAAAT TGCCGTCAAC 3060

CAAGCTCTGA TAGAGTTGGT CAAGACCAAT GCGGAGCATA TACGCCCGGA GCCGCGGCGA 3120

TCCTGCAAGC TCCGGATGCC TCCGCTCGAA GTAGCGCGTC TGCTGCTCCA TACAAGCCAA 3180

CCACGGCCTC CAGAAGAAGA TGTTGGCGAC CTCGTATTGG GAATCCCCGA ACATCGCCTC 3240

GCTCCAGTCA ATGACCGCTG TTATGCGGCC ATTGTCCGTC AGGACATTGT TGGAGCCGAA 3300

ATCCGCGTGC ACGAGGTGCC GGACTTCGGG GCAGTCCTCG GCCCAAAGCA TCAGCTCATC 3360

GAGAGCCTGC GCGACGGACG CACTGACGGT GTCGTCCATC ACAGTTTGCC AGTGATACAC 3420

ATGGGGATCA GCAATCGCGC ATATGAAATC ACGCCATGTA GTGTATTGAC CGATTCCTTG 3480

CGGTCCGAAT GGGCCGAACC CGCTCGTCTG GCTAAGATCG GCCGCAGCGA TCGCATCCAT 3540

GGCCTCCGCG ACCGGCTGCA GAACAGCGGG CAGTTCGGTT TCAGGCAGGT CTTGCAACGT 3600

GACACCCTGT GCACGGCGGG AGATGCAATA GGTCAGGCTC TCGCTGAATT CCCCAATGTC 3660

AAGCACTTCC GGAATCGGGA GCGCGGCCGA TGCAAAGTGC CGATAAACAT AACGATCTTT 3720

GTAGAAACCA TCGGCGCAGC TATTTACCCG CAGGACATAT CCACGCCCTC CTACATCGAA 3780

GCTGAAAGCA CGAGATTCTT CGCCCTCCGA GAGCTGCATC AGGTCGGAGA CGCTGTCGAA 3840

CTTTTCGATC AGAAACTTCT CGACAGACGT CGCGGTGAGT TCAGGCTTTT TCATATCTCA 3900

TTGCCCCCGG ACGAGGATCT GCGGCACGCT GTTGACGCTG TTAAGCGGGT CGCTGCAGGG 3960

TCGCTCGGTG TTCGAGGCCA CACGCGTCAC CTTAATATGC GAAGTGGACC TGGGACCGCG 4020

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CGAGCAACGG GCCACGGGGA TGAAGCAGGG CGGCACCTCG CTAACGGATT CACCACTCCA 4200

AGAATTGGAG CCAATCAATT CTTGCGGAGA ACTGTGAATG CGCAAACCAA CCCTTGGCAG 4260

AACATATCCA TCGCGTCCGC CATCTCCAGC AGCCGCACGC GGCGCATCTC GGGCAGCGTT 4320

GGGTCCTGGC CACGGGTGCG CATGATCGTG CTCCTGTCGT TGAGGACCCG GCTAGGCTGG 4380

CGGGGTTGCC TTACTGGTTA GCAGAATGAA TCACCGATAC GCGAGCGAAC GTGAAGCGAC 4440

TGCTGCTGCA AAACGTCTGC GACCTGAGCA ACAACATGAA TGGTCTTCGG TTTCCGTGTT 4500

TCGTAAAGTC TGGAAACGCG GAAGTCAGCG CTCTTCCGCT TCCTCGCTCA CTGACTCGCT 4560

GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT 4620

ATCCACAGAA TCAGGGGATA ACGCAGGAAA GAACATGTGA GCAAAAGGCC AGCAAAAGGC 4680

CAGGAACCGT AAAAAGGCCG CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA 4740

GCATCACAAA AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA 4800

CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC TGCCGCTTAC 4860

CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCATA GCTCACGCTG 4920

TAGGTATCTC AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC ACGAACCCCC 4980

CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG 5040

ACACGACTTA TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT 5100

AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT 5160

ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG 5220

ATCCGGCAAA CAAACCACCG CTGGTAGCGG TGGTTTTTTT GTTTGCAAGC AGCAGATTAC 5280

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GTGGAACGAA AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC 5400

CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC 5460

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TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC GGGAGGGCTT 5580

ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT 5640

ATCAGCAATA AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC 5700

CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA 5760

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TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT 5880

GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC 5940

AGTGTTATCA CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT 6000

AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG 6060

GCGACCGAGT TGCTCTTGCC CGGCGTCAAC ACGGGATAAT ACCGCGCCAC ATAGCAGAAC 6120

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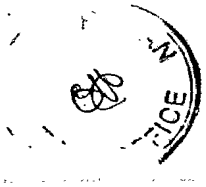

TACTTTCACC AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG 6300

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ACAAATAGGG GTTCCGCGCA CATTTCCCCG AAAAGTGCCA CCTGACGTCT AAGAAACCAT 6480

TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG AGGCCCTTTC GTCTTCAAGA 6540

ATTCTCATGT TTGACAGCTT ATCGATCCGG CCAACGGTGT TGCCATTGCT GCAGGCGCAG 6600

AGCTGGTAGG TATGGAAGAT CTATACAGTG 6630

S014668C.58

Claims

1. Process for determining the modulating effect of a substance on a receptor-dependent signal transduction pathway in a human or animal cell, characterised in that the modulating effect of the substance on the activity of a phospholipase or on a mechanism which precedes or succeeds the phospholipase activation in the signal transduction pathway initiated by a receptor coupled to the signal transduction pathway, is determined by incubating mammalian cells which

a) are transformed with a recombinant DNA containing a reporter gene and a regulatory sequence which responds to the change in concentration of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) brought about by the modulation of the phospholipase activity, so that the expression of the reporter gene is modulated by a change in concentration of IP_3/DAG, and which are furthermore

b) transformed with a recombinant DNA, containing a sequence which codes for a receptor which is coupled to the phospholipase effector system so that the cells express the receptor,

with the test substance and measuring the concentration of the reporter gene product.

2. Process according to claim 1, characterised in that the recombinant DNA defined in b) contains a sequence which codes for a human receptor.

3. Process according to claim 1 or 2, characterised in that the recombinant DNA defined in a) contains a regulatory sequence which responds to the change in concentration of IP_3 and DAG brought about by modulation of phospholipase C, and that the recombinant DNA defined

in b) contains a sequence which codes for a G-protein coupled receptor.

4. Process according to one of claims 1 to 3, characterised in that, furthermore, mammalian cells which are transformed only with the recombinant DNA defined in a) are incubated with the test substance under identical conditions and the concentration of the reporter gene product is measured.

5. Process according to one of claims 1 to 4, characterised in that, moreover, mammalian cells which are transformed with recombinant DNA containing the reporter gene and a regulatory sequence which responds to the change in concentration of cAMP brought about by the modulation of adenylate cyclase so that the expression of the reporter gene is modulated by the change in concentration of cAMP are incubated with the test substance and the concentration of the reporter gene product is measured.

6. Process according to claim 5, characterised in that, moreover, cells which are transformed with the recombinant DNA responding to cAMP and with a recombinant DNA defined in b), containing the sequence coding for the same receptor as the cells which are transformed with recombinant DNA responding to IP_3/DAG, are incubated with the test substance and the concentration of the reporter gene product is measured.

7. Process according to claim 5, characterised in that, moreover, cells which are transformed with a recombinant DNA, containing a sequence coding for a receptor which is coupled to the adenylate cyclase effector system in such a way that the cells express the receptor, are incubated with the test substance and the concentration of the reporter gene product is measured.

8. Process according to one of claims 1 to 7, characterised in that it is used as a screening assay in which the test substance is one of a number of substances with which a predetermined number of mammalian cells are incubated under predetermined conditions and the concentration of the reporter gene product is measured.

9. Process according to claim 8, characterised in that, as the reporter gene product, luciferase is measured in the presence of a reagent which contains a detergent suitable for lysing the cells, a buffer having a pH from 6 to 9, preferably pH 7.8, a magnesium salt, preferably magnesium sulphate, adenosine triphosphate, luciferin, a mild organic reducing agent such as dithiothreitol and/or β-mercaptoethanol and optionally sodium tripolyphosphate and/or sodium pyrophosphate.

10. Reagent when used for carrying out the process according to claim 9, characterised in that it contains a detergent suitable for lysing the cells, a buffer with a pH of 6 to 9, preferably 7.8, a magnesium salt, preferably magnesium sulphate, adenosine triphosphate, luciferin, a mild organic reducing agent such as dithiothreitol and/or β-mercaptoethanol and optionally sodium tripolyphosphate and/or sodium pyrophosphate.

11. Recombinant DNA containing a reporter gene and an expression control sequence functionally connected therewith for the expression in mammalian cells, characterised in that the expression control sequence contains a regulatory sequence which responds to the change in concentration of IP_3 and DAG brought about by the modulation of phospholipase C, when used in a process according to one of the preceding claims.

12. Recombinant DNA according to claim 11,

characterised in that it contains a regulatory sequence of natural origin.

13. Recombinant DNA according to claim 12, characterised in that it contains the 5'-regulatory sequence of a gene which can be induced by IP_3/DAG.

14. Recombinant DNA according to claim 13, characterised in that it contains the 5'-regulatory sequence of the ICAM-1 gene.

15. Recombinant DNA according to claim 11, characterised in that the regulatory sequence is synthetically produced.

16. Recombinant DNA according to claim 15, characterised in that the regulatory sequence contains a plurality of regulatory elements responding to the modulation of IP_3/DAG spaced from one another.

17. Recombinant DNA according to claim 16, characterised in that it contains three to twelve regulatory elements.

18. Recombinant DNA according to claim 16 or 17, characterised in that at least some of the regulatory elements and/or the sequence sections located between them differ from one another.

19. Recombinant DNA according to claim 17 or 18, characterised in that it contains the TRE-elements of the ICAM-1 gene.

20. Recombinant DNA according to claim 17 or 18, characterised in that it contains six TRE-elements of the ICAM-1 gene.

21. Recombinant DNA according to one of claims 11 to 20, characterised in that it contains a luciferase gene as reporter gene.

22. Recombinant DNA according to one of claims 11 to 21, characterised in that it contains a marker gene.

23. Mammalian cells transformed with a recombinant DNA according to one of claims 11 to 22.

24. Mammalian cells according to claim 23, characterised in that they are moreover transformed with a recombinant DNA containing a sequence coding for a receptor which is coupled to the phospholipase C-effector system in such a way that the cells express the receptor.

25. Mammalian cells according to claim 24, characterised in that the recombinant DNA contains a sequence coding for a human receptor.

26. Mammalian cells according to claim 24 or 25, characterised in that the recombinant DNA contains a sequence coding for a G-protein-coupled receptor.

27. Mammalian cells according to claim 26, characterised in that the expression control sequence contains a regulatory sequence which responds to the change in concentration of IP_3 and DAG caused by the modulation of phospholipase C.

28. Mammalian cells according to claim 27, characterised in that they are transformed with recombinant DNA containing a sequence coding for a receptor, selected from the group comprising serotonin receptors of the 5-HT_{1C}- and 5-HT_2-type, thrombin receptor, neuropeptide Y-receptors, neurokinin

receptors, PAF-receptors, angiotensin receptors, muscarinic acetylcholine receptors of the M_1-, M_3- and M_5-type.

29. Recombinant DNA, containing a reporter gene and an expression control sequence functionally connected therewith for expression in mammalian cells, wherein the expression control sequence contains a synthetically produced regulatory sequence which responds to the change in concentration of cAMP brought about by modulation of adenylate cyclase, characterised in that the regulatory sequence contains a plurality of regulatory elements responding to the modulation of cAMP, spaced apart from one another, when used in a process according to one of claims 5 to 9.

30. Recombinant DNA according to claim 29, characterised in that it contains three to twelve regulatory elements.

31. Recombinant DNA according to claim 29 or 30, characterised in that at least some of the regulatory elements and/or the sequence sections located between them differ from one another.

32. Recombinant DNA according to one of claims 29 to 31, characterised in that it contains a luciferase gene as the reporter gene.

33. Recombinant DNA according to one of claims 29 to 32, characterised in that it contains a marker gene.

34. Mammalian cells transformed with a recombinant DNA according to one of claims 29 to 33.

35. Mammalian cells according to claim 34, characterised in that they are furthermore transformed with a recombinant DNA containing a sequence coding for a receptor which is coupled to the phospholipase C-effector system, so that the cells express the receptor.

characterised in that they are transformed with recombinant DNA containing a sequence coding for a receptor selected from the group comprising serotonin receptors of the 5-HT_{1C}- and 5-HT_2-type, thrombin receptor, neuropeptide Y-receptors, neurokinin receptors, PAF-receptors, angiotensin receptors and muscarinic acetylcholine receptors of the M_1-, M_3- and M_5-type.

37. Mammalian cells according to claim 34, characterised in that they are also transformed with a recombinant DNA containing a sequence coding for a receptor which is coupled to the adenylate cyclase effector system, in such a way that the cells express the receptor.

38. Mammalian cells according to claim 37, characterised in that they are transformed with recombinant DNA containing a sequence coding for a receptor, selected from the group comprising muscarinic acetylcholine receptors of the M_2- and M_4-type, dopamine receptors of the D_1-, D_{21}-, D_{2s}- and D_5-type, serotonin receptors of the 5-HT_{1A}- and 5-HT_{1D}-type and adenosine receptors of the A_1- and A_2-type.

39. A process for determining the modulating effect of a substance on a receptor-dependent signal transduction pathway according to any one of claims 1 to 9 substantially as hereinbefore described, with particular reference to any one of Examples 1 to 8 and the Figures.

40. A reagent when used for carrying out the process according to claim 10 substantially as hereinbefore described, with particular reference to Example 9.

41. Recombinant DNA containing a reporter gene and an expression control sequence functionally connected therewith for the expression in mammalian cells,

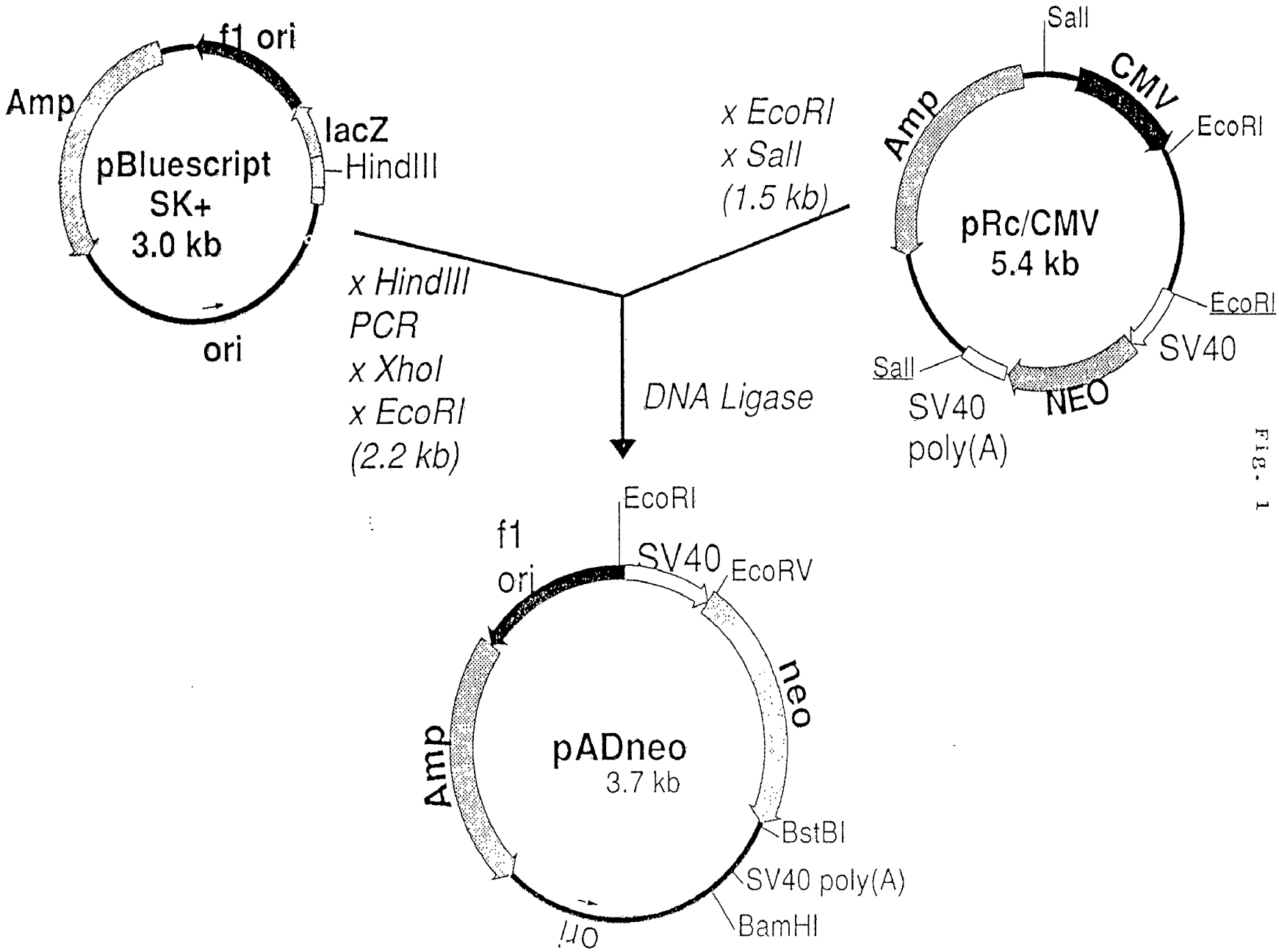
according to any one of claims 11 to 22 or 29 to 33, substantially as hereinbefore described, with particular reference to Example 1 or 2 and the Figures.

42. Mammalian cells transformed with a recombinant DNA according to any one of claims 23 to 28 or 34 to 38 substantially as hereinbefore described, with particular reference to any one of Examples 6 to 8.

Dated this 12th day of February, 1996

Boehringer Ingelhem International GmbH

By its Patent Attorneys
Davies Collison Cave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pBluescript
SK+
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ori
x HindIII
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x XhoI
x EcoRI
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x EcoRI
x SalI
(1.5 kb)
DNA Ligase
SalI
CMV
Amp
EcoRI
pRc/CMV
5.4 kb
EcoRI
SV40
SalI
SV40
poly(A)
NEO
EcoRI
f1
ori
SV40
EcoRV
neo
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BamHI
ori

Fig. 1

BglII - BamHI Fragment after *PCR* of *pX1*

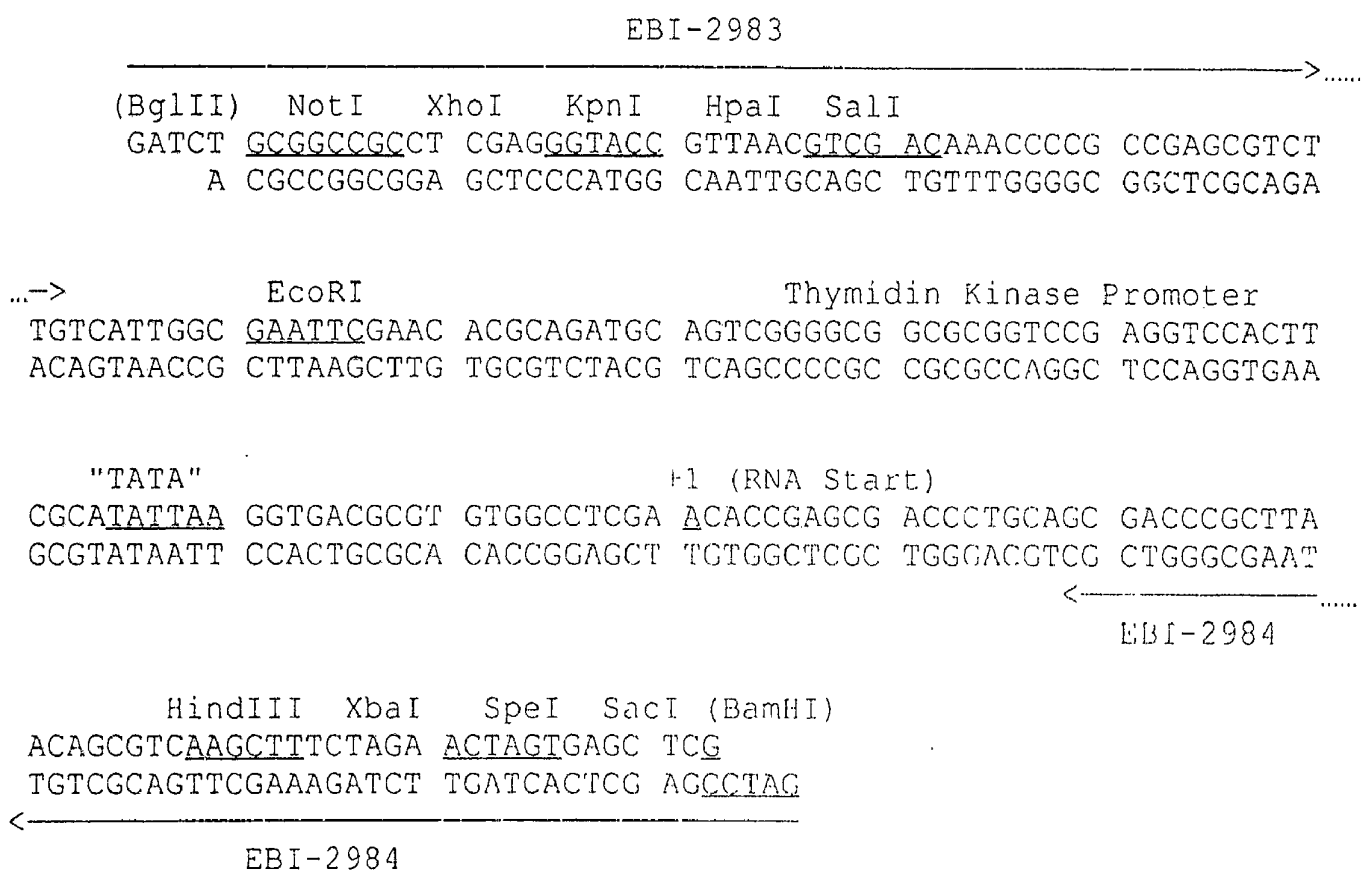
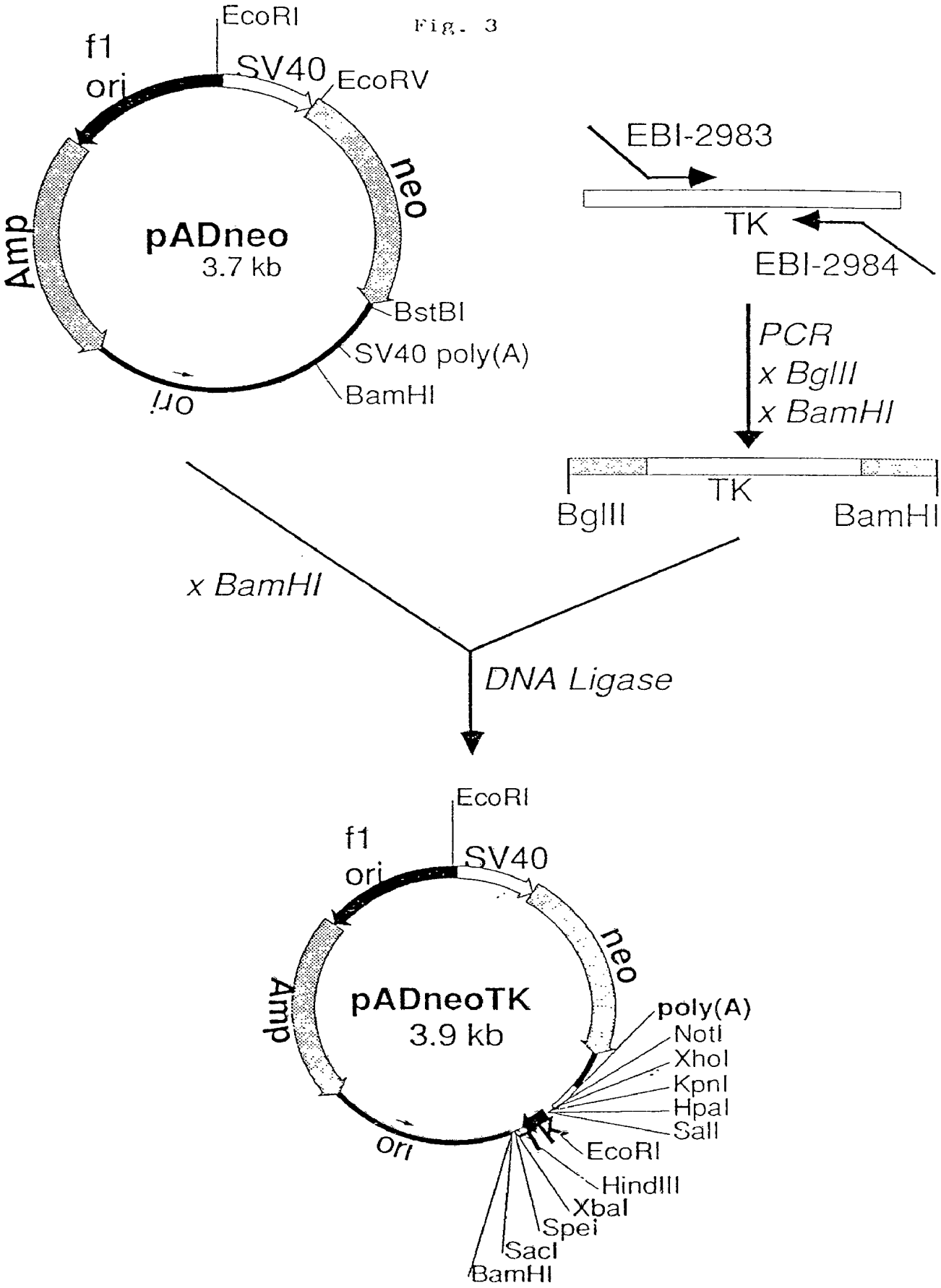


Fig. 2

Fig. 3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SV40
EcoRV
neo
Amp
pADneo
3.7 kb
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BamHI
ori
EBI-2983
TK
EBI-2984
PCR
x BglII
x BamHI
TK
BglII
BamHI
x BamHI
DNA Ligase
EcoRI
f1
ori
SV40
neo
Amp
pADneoTK
3.9 kb
poly(A)
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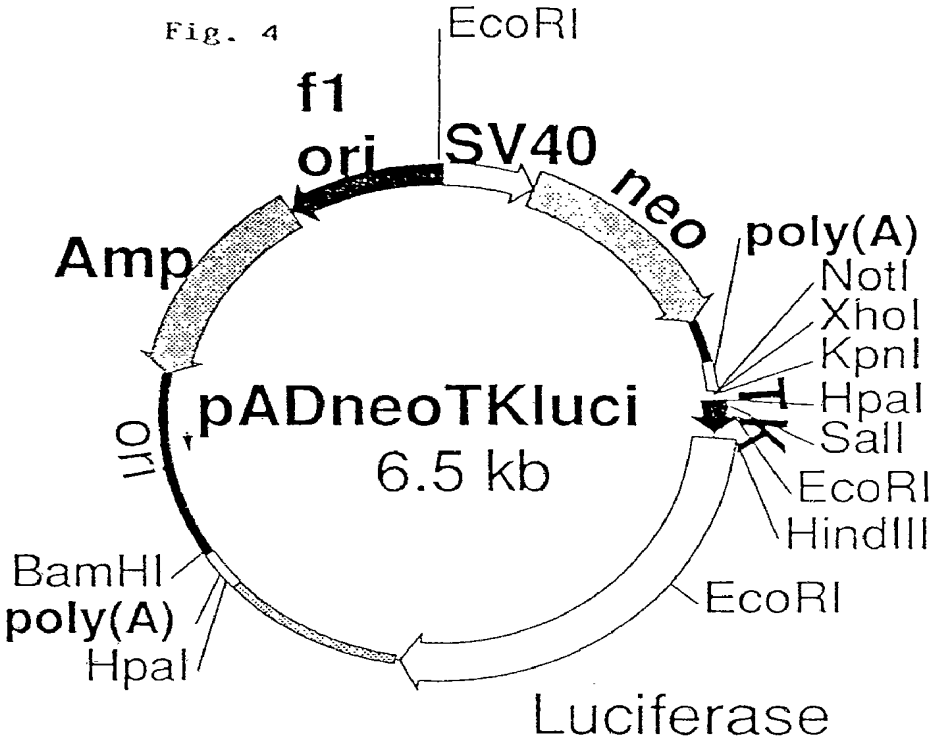
KpnI
HpaI
SalI
TK
EcoRI
HindIII
XbaI
SpeI
SacI
BamHI
ori

Fig. 4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SV40
neo
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HpaI
SalI
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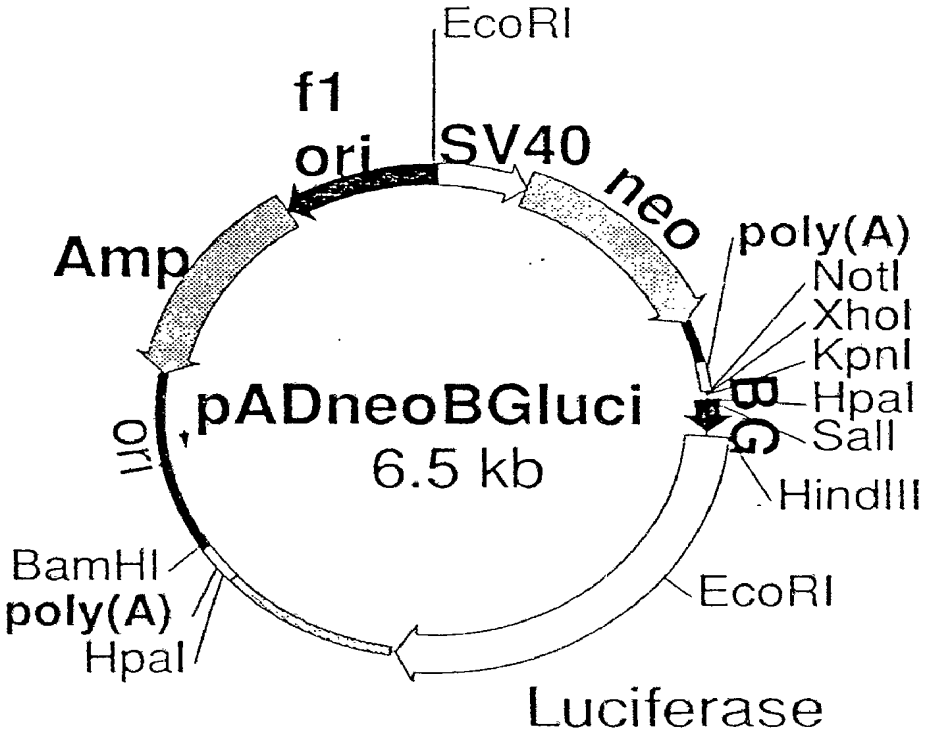
TK
ori
BamHI
poly(A)
HpaI
Luciferase

A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KpnI
HpaI
SalI
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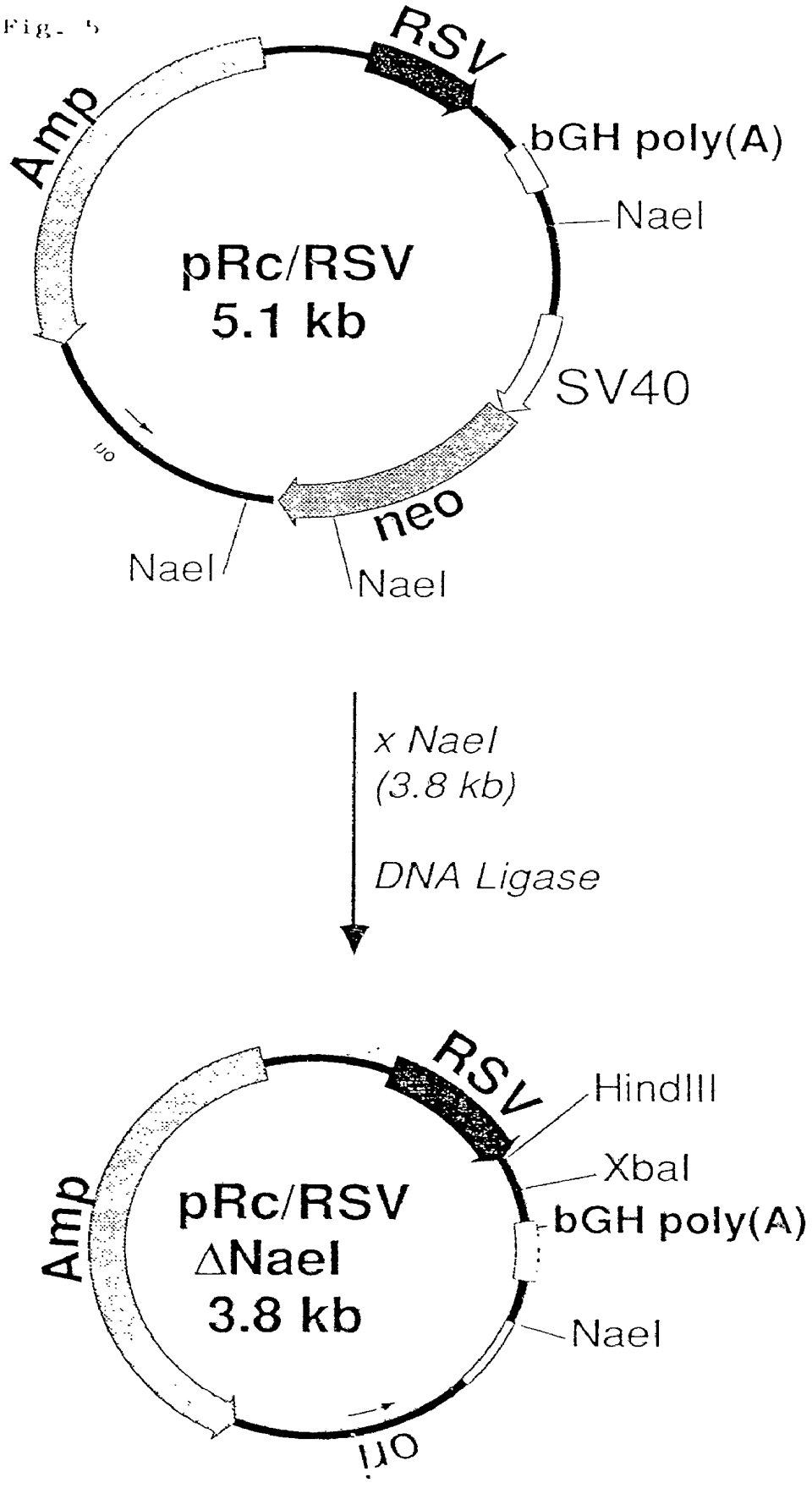
BG
ori
BamHI
poly(A)
HpaI
Luciferase

B

Fig. 5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NaeI
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SV40
Amp
ori
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NaeI
NaeI
x NaeI
(3.8 kb)
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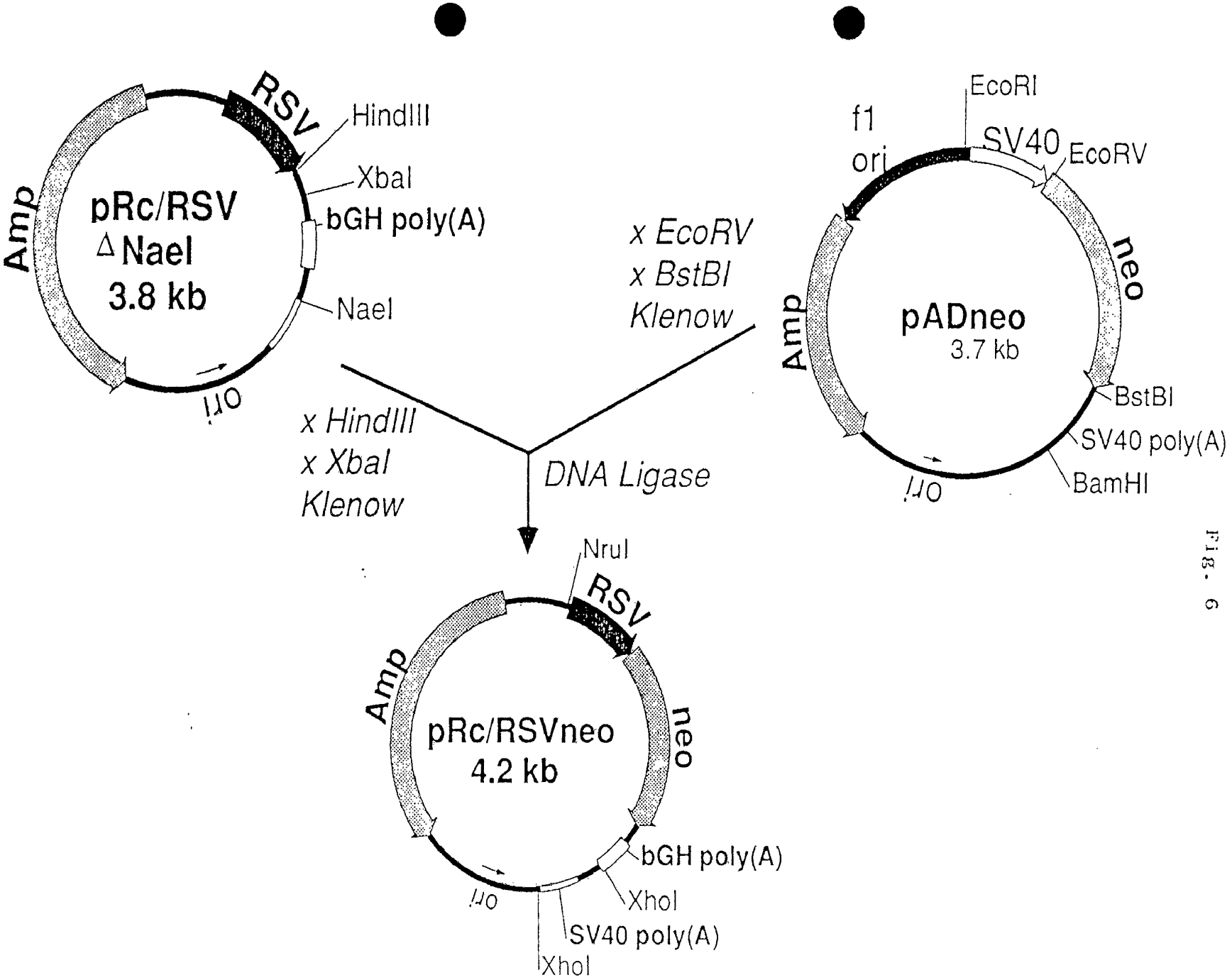
HindIII
XbaI
bGH poly(A)
pRc/RSV
ΔNaeI
3.8 kb
Amp
NaeI
ori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x BstBI
Klenow
x HindIII
x XbaI
Klenow
DNA Ligase
EcoRI
f1
ori
SV40
EcoRV
neo
Amp
pADneo
3.7 kb
BstBI
SV40 poly(A)
BamHI
ori
NruI
RSV
Amp
pRc/RSVneo
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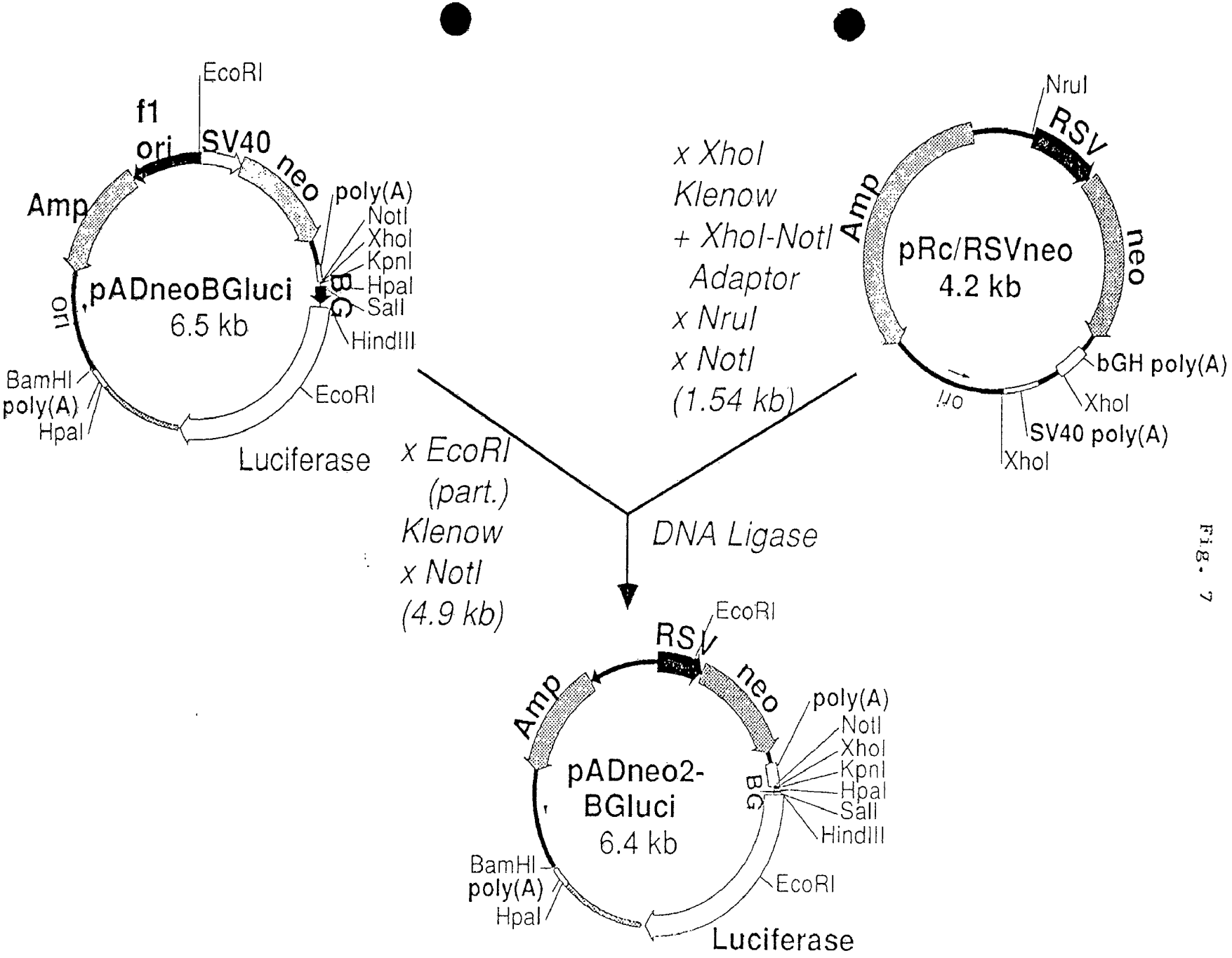
neo
bGH poly(A)
XhoI
ori
SV40 poly(A)
XhoI

Fig. 6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SV40
neo
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NotI
XhoI
KpnI
HpaI
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Amp
pADneoBGluci
6.5 kb
ori
BG
BamHI
poly(A)
HpaI
EcoRI
Luciferase
x XhoI
Klenow
+ XhoI-NotI
Adaptor
x NruI
x NotI
(1.54 kb)
NruI
RSV
Amp
pRc/RSVneo
4.2 kb
neo
bGH poly(A)
ori
XhoI
SV40 poly(A)
XhoI
x EcoRI
(part.)
Klenow
x NotI
(4.9 kb)
DNA Ligase
EcoRI
RSV
neo
Amp
poly(A)
NotI
XhoI
KpnI
HpaI
SalI
HindIII
pADneo2-
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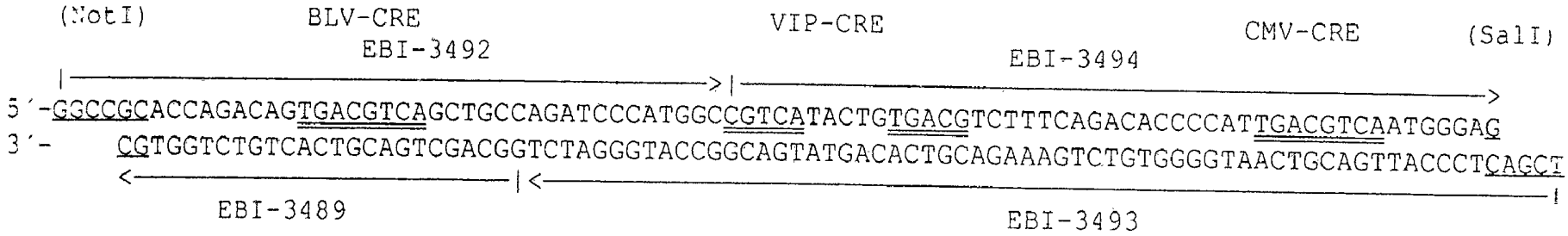
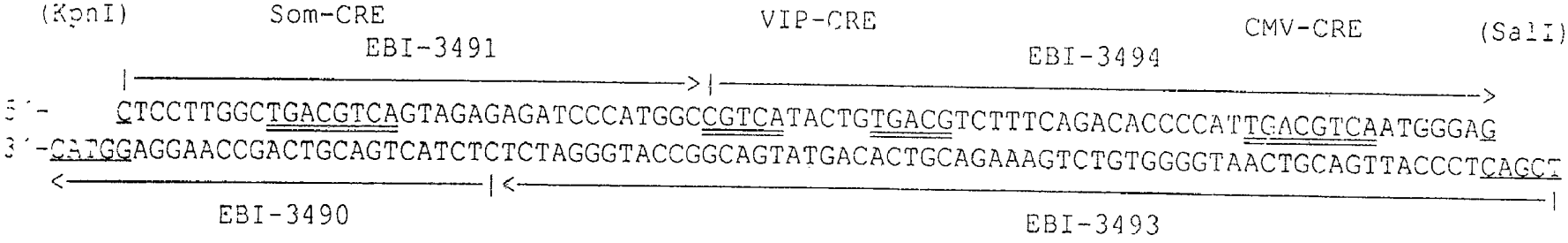
HpaI
EcoRI
Luciferase

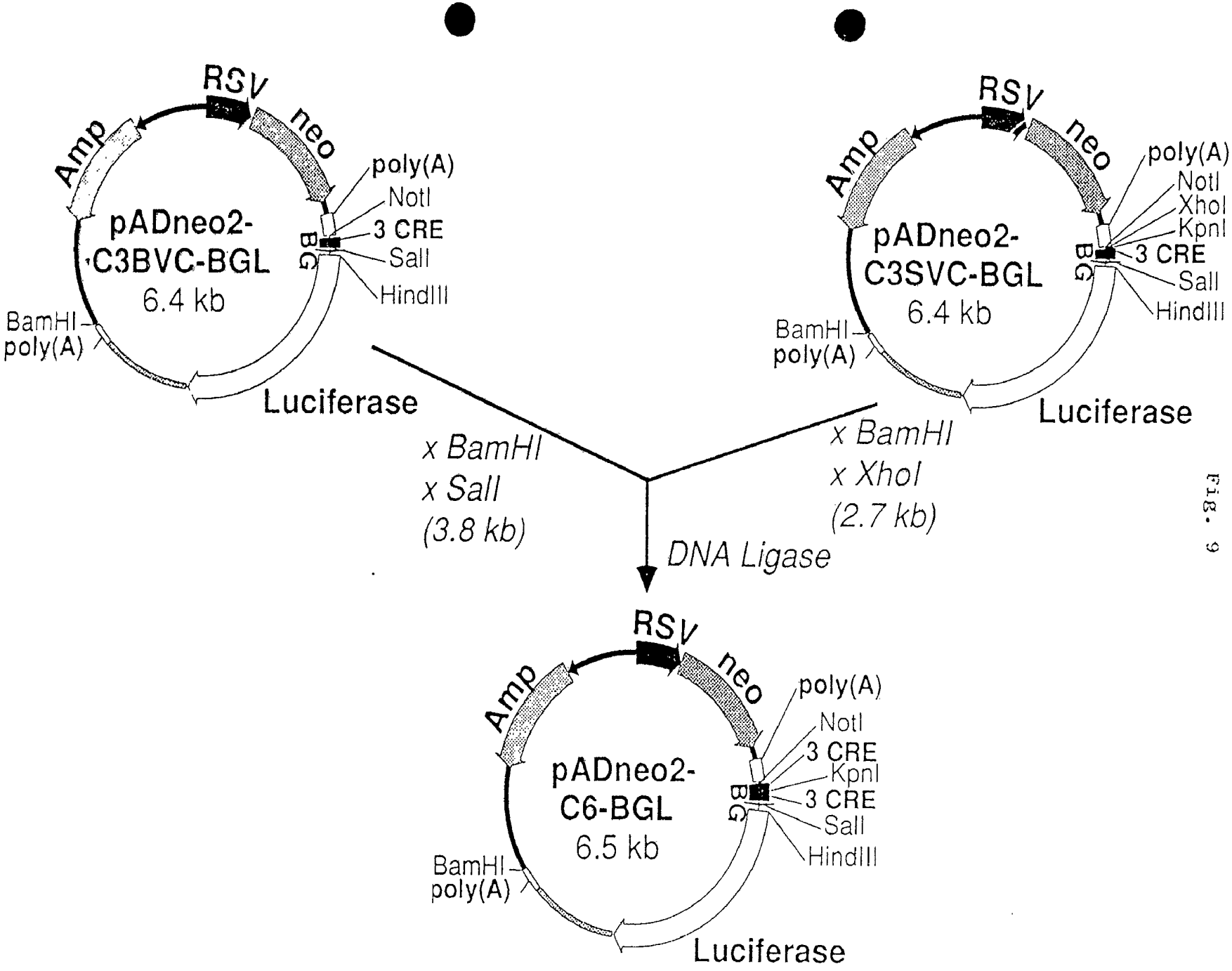
Fig. 7

CRE - OLIGONUCLEOTIDES

FIG. 8



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NotI
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BG
SalI
HindIII
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BamHI
poly(A)
Luciferase
x BamHI
x SalI
(3.8 kb)
RSV
neo
Amp
poly(A)
NotI
XhoI
KpnI
3 CRE
BG
SalI
HindIII
pADneo2-
C3SVC-BGL
6.4 kb
BamHI
poly(A)
Luciferase
x BamHI
x XhoI
(2.7 kb)
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Amp
poly(A)
NotI
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KpnI
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poly(A)
Luciferase

Fig. 9

Fig. 10

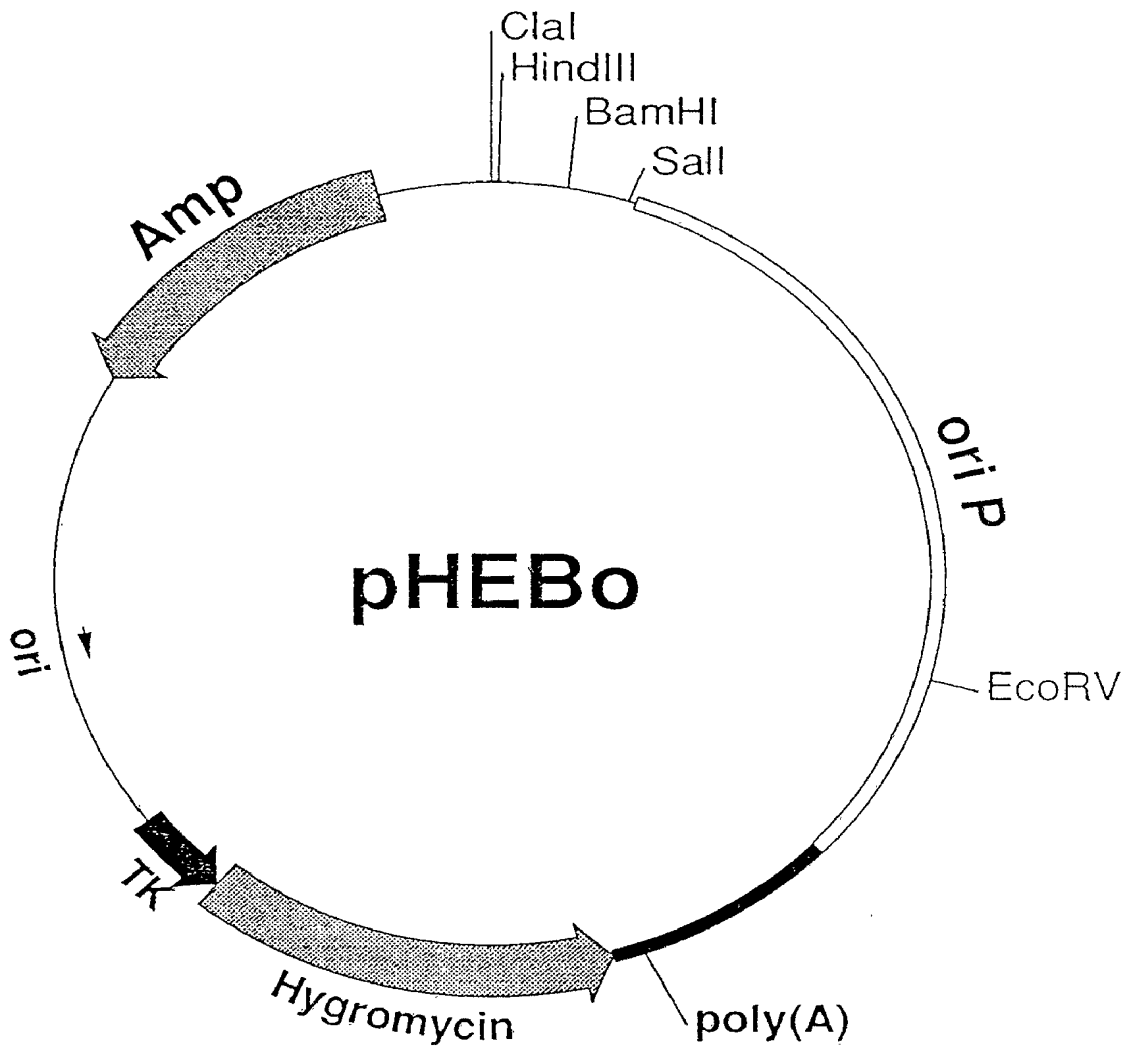
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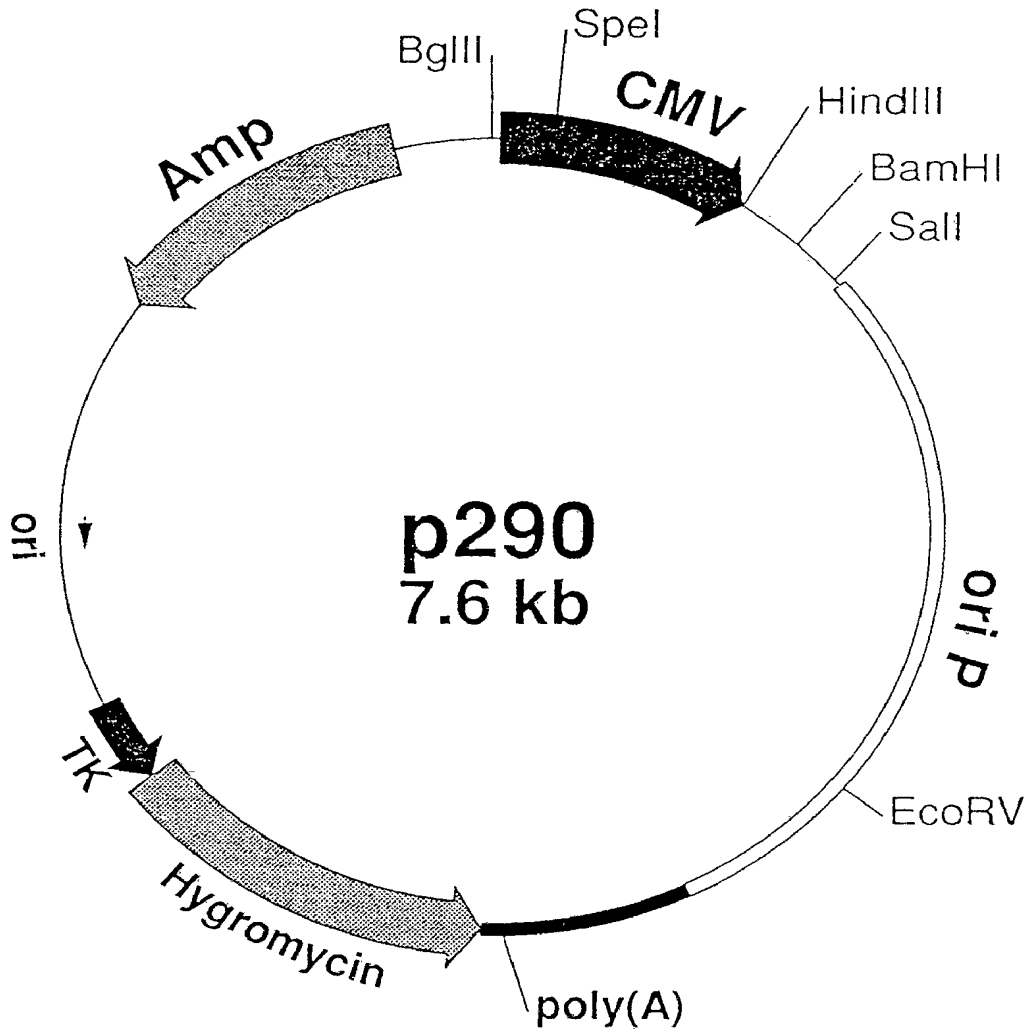
Fig. 11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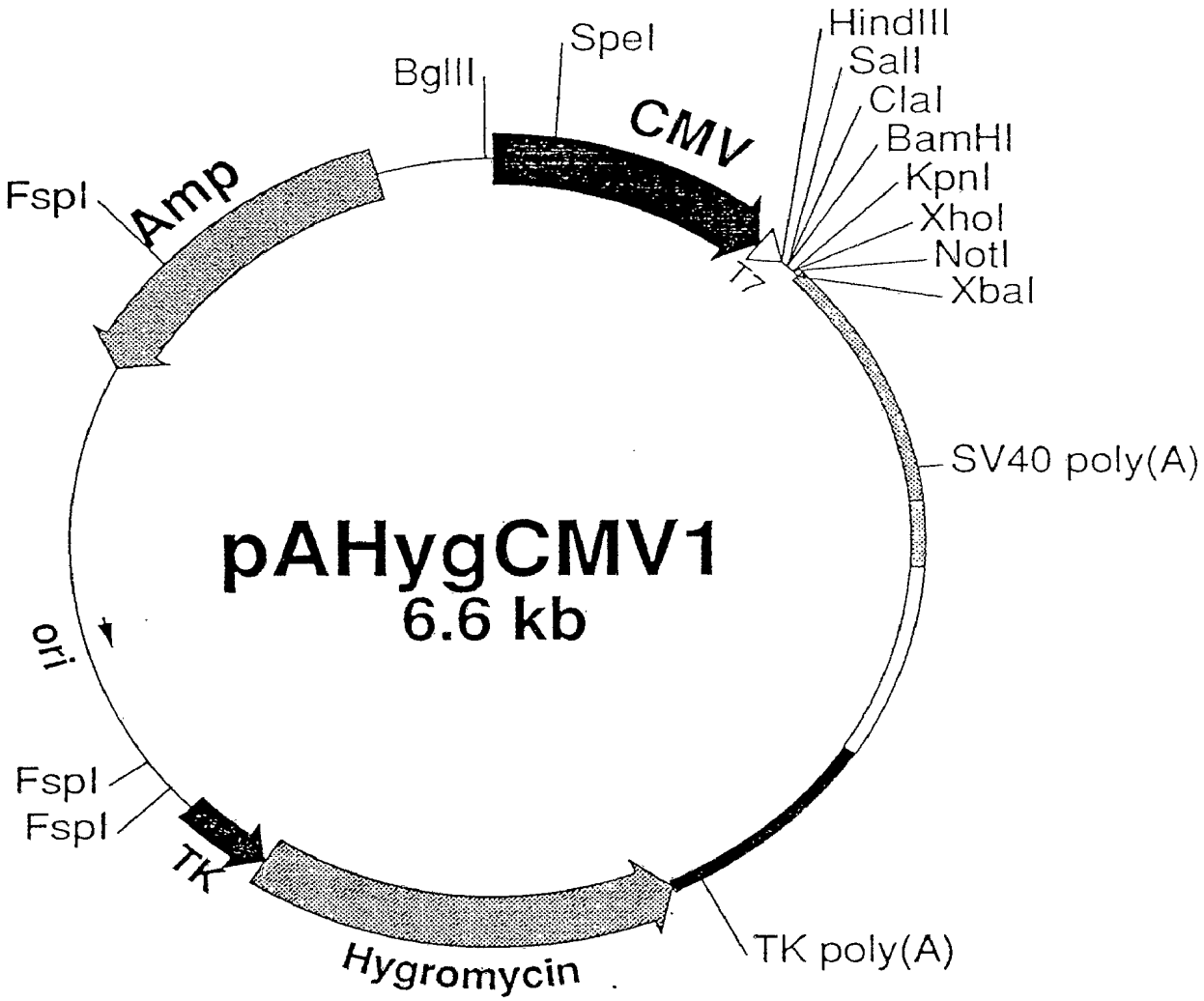
poly(A)

Fig. 12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FspI
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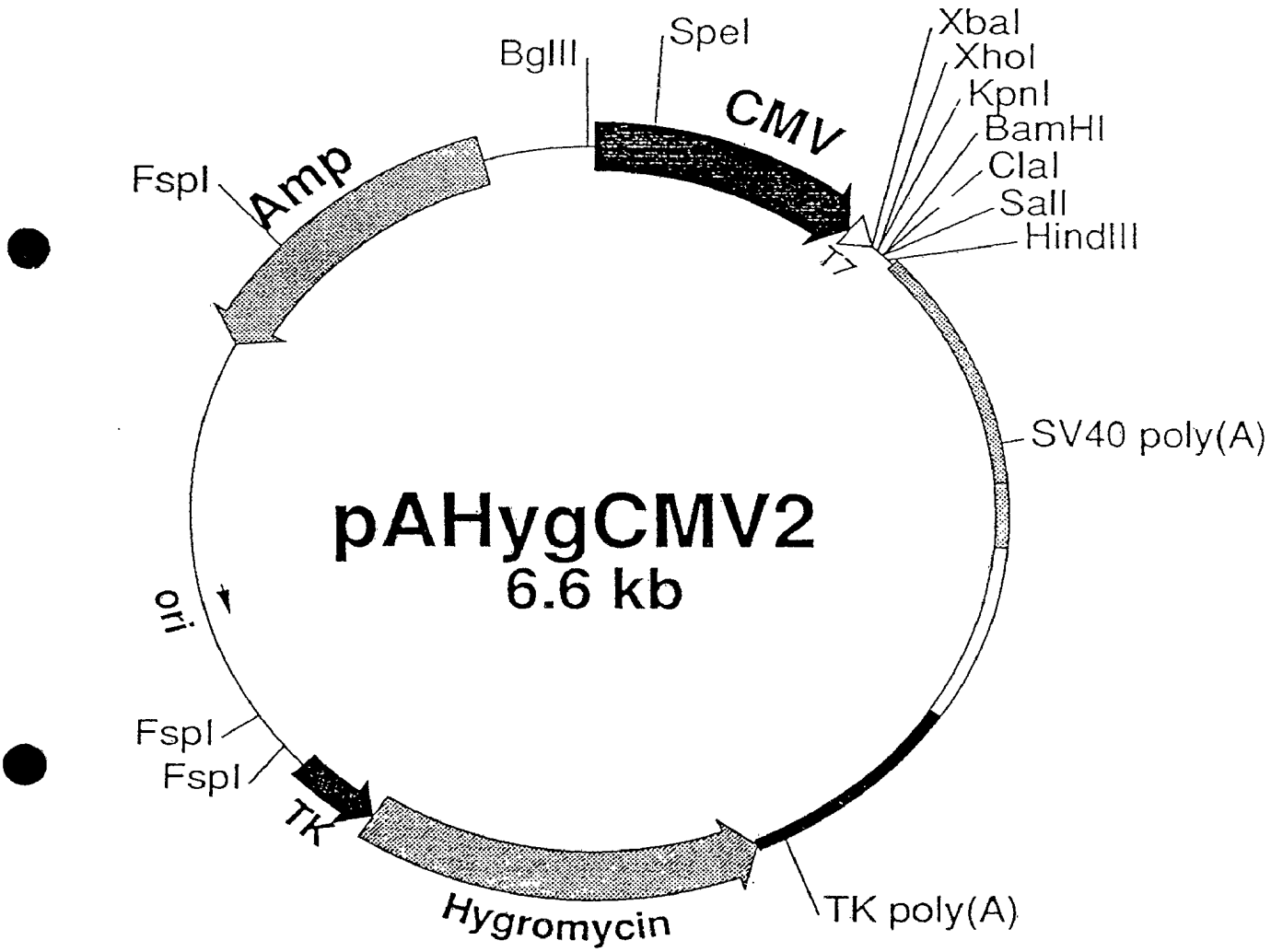
TK
Hygromycin
TK poly(A)

Fig. 13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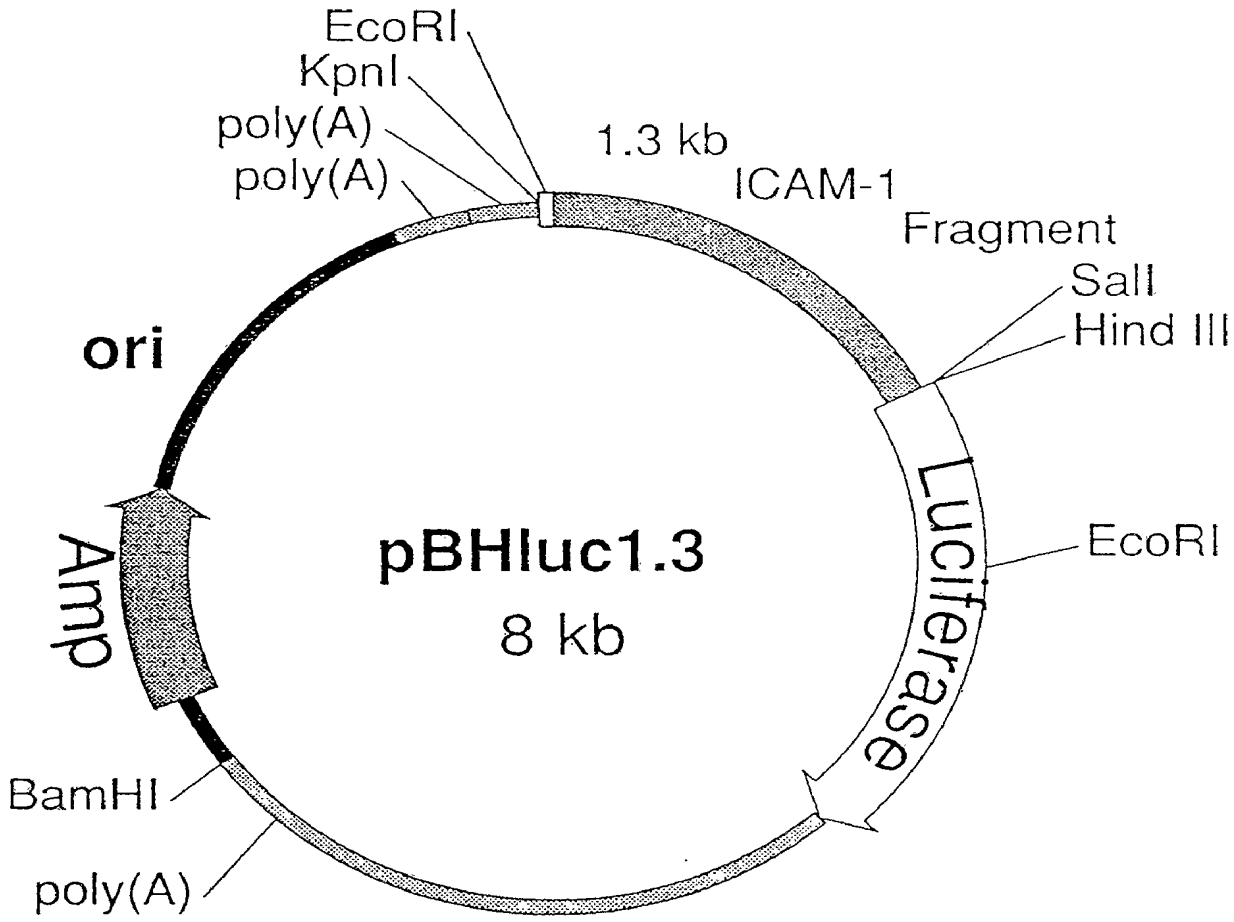
Hygromycin
TK poly(A)

Fig. 14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poly(A)
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Fragment
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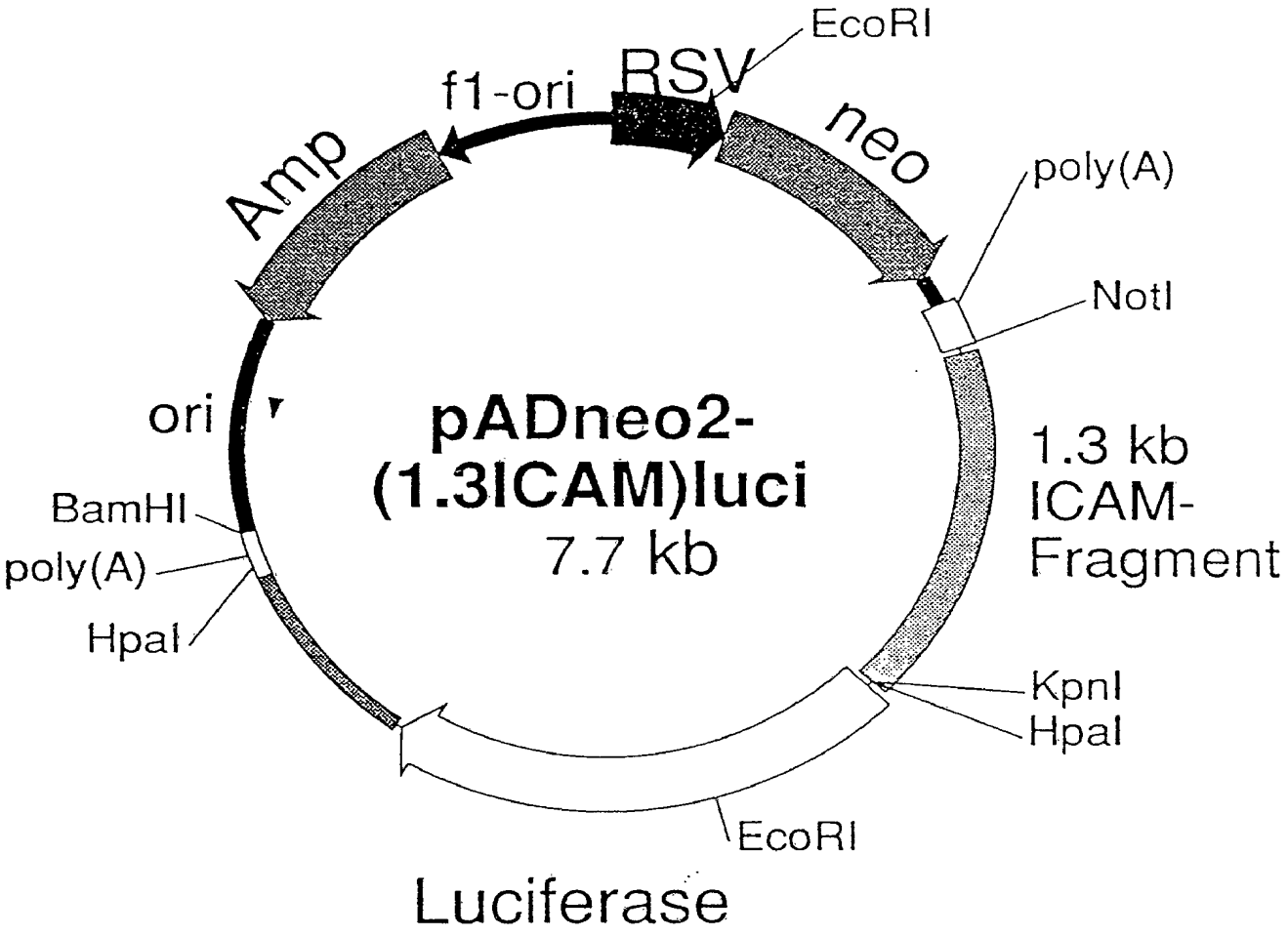
Hind III
ori
Luciferase
EcoRI
pBHluc1.3
8 kb
Amp
BamHI
poly(A)

Fig. 15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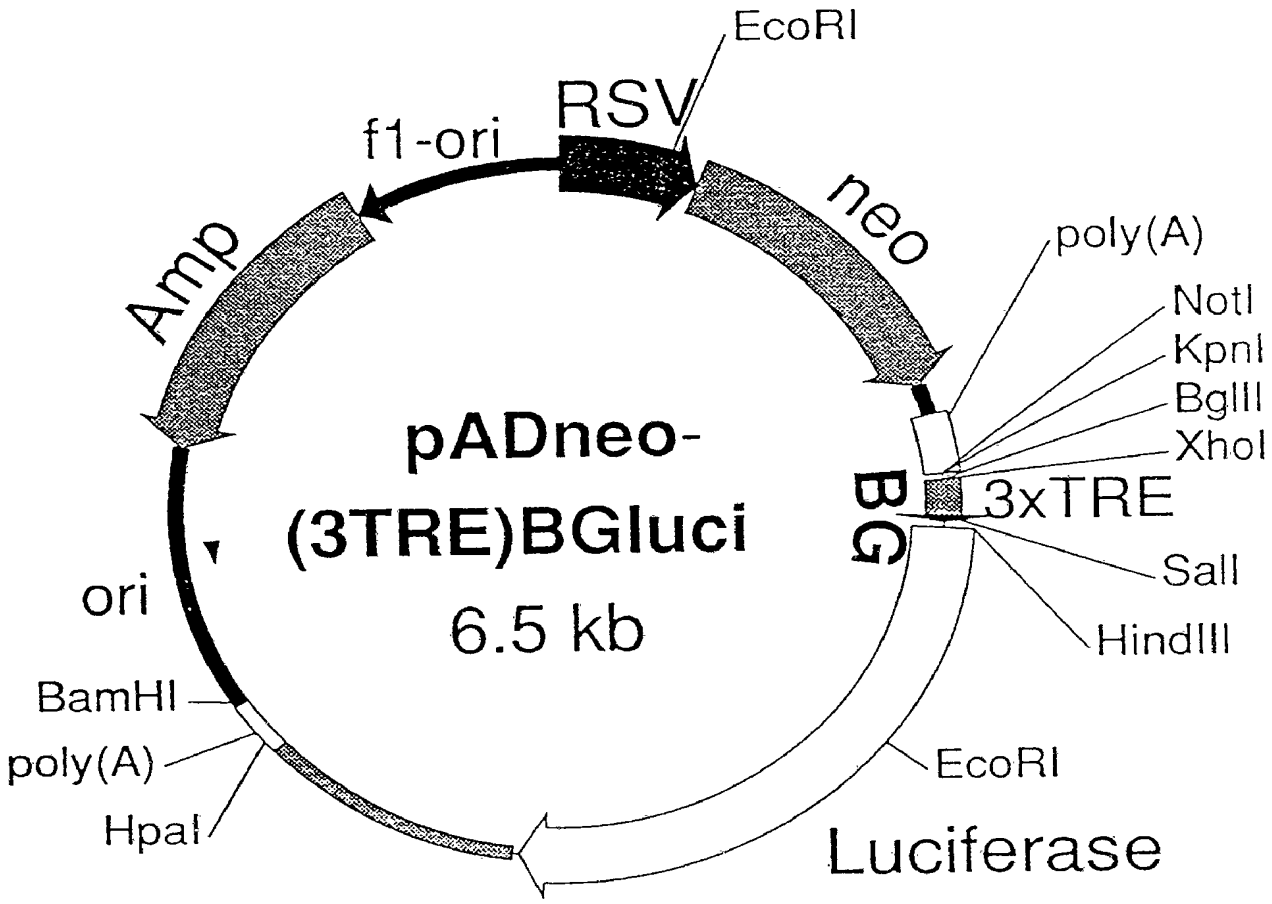
Fragment
BamHI
poly(A)
HpaI
KpnI
HpaI
EcoRI
Luciferase

Fig. 16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Amp
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NotI
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BglII
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3xTRE
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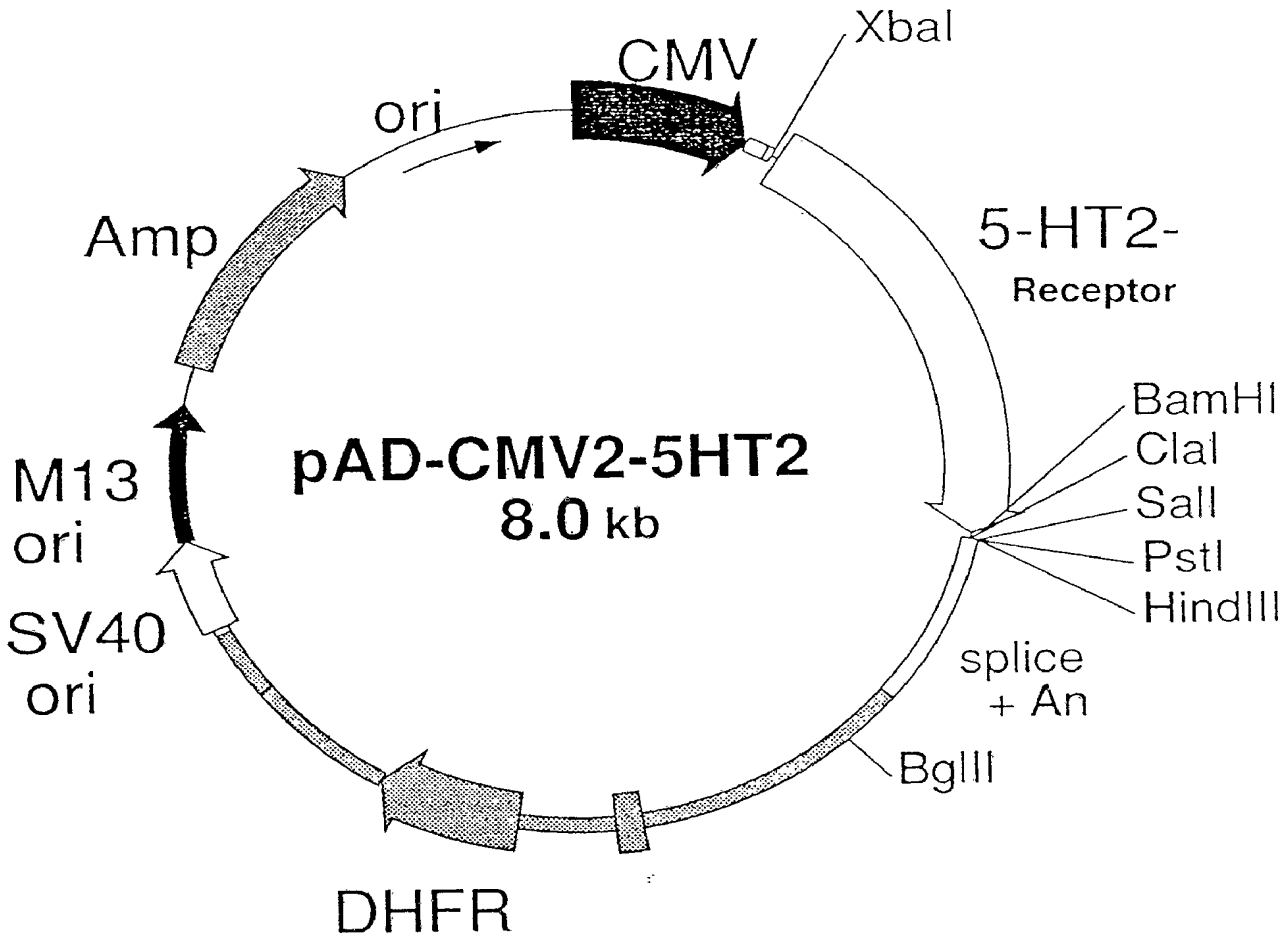
ori
BamHI
poly(A)
HpaI
EcoRI
Luciferase

Fig. 17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XbaI
ori
Amp
5-HT2-
Receptor
BamHI
ClaI
SalI
PstI
HindIII
pAD-CMV2-5HT2
8.0 kb
M13
ori
SV40
ori
splice
+ An
BglII
DHFR

Fig. 18

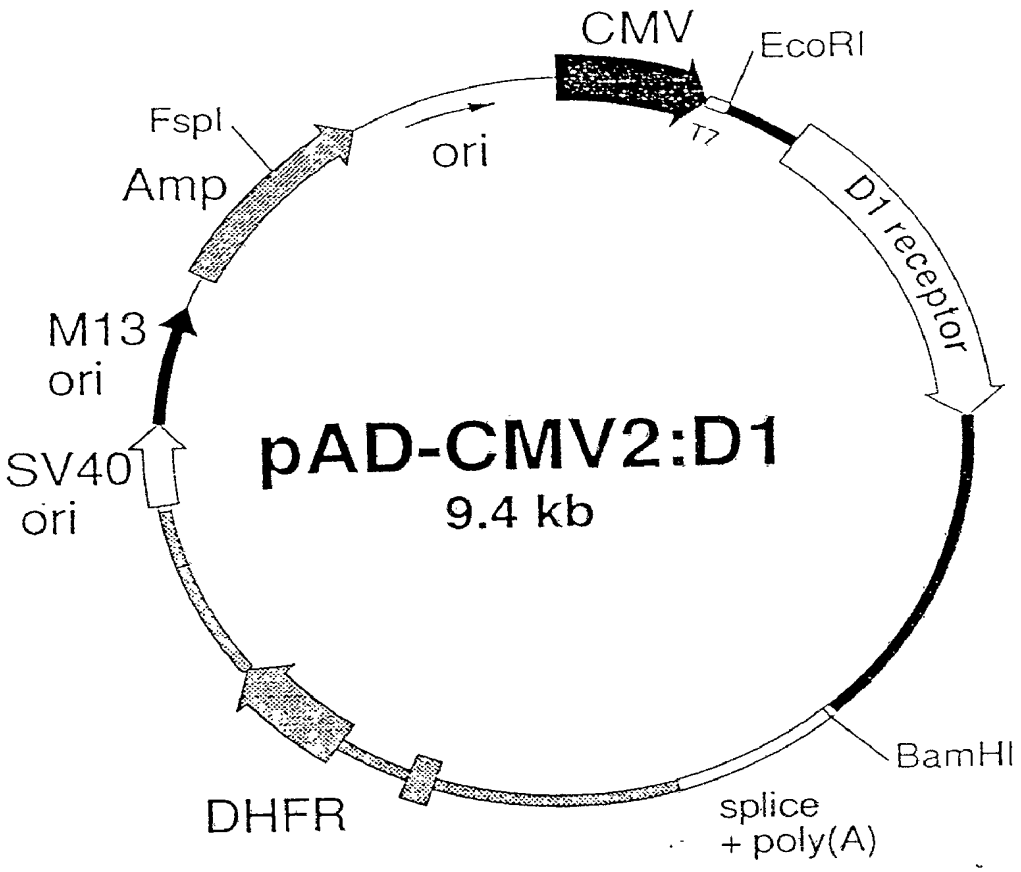

CMV
EcoRI
T7
D1 receptor
BamHI
splice
+ poly(A)
DHFR
SV40
ori
M13
ori
Amp
FspI
ori
pAD-CMV2:D1
9.4 kb

Fig. 19A

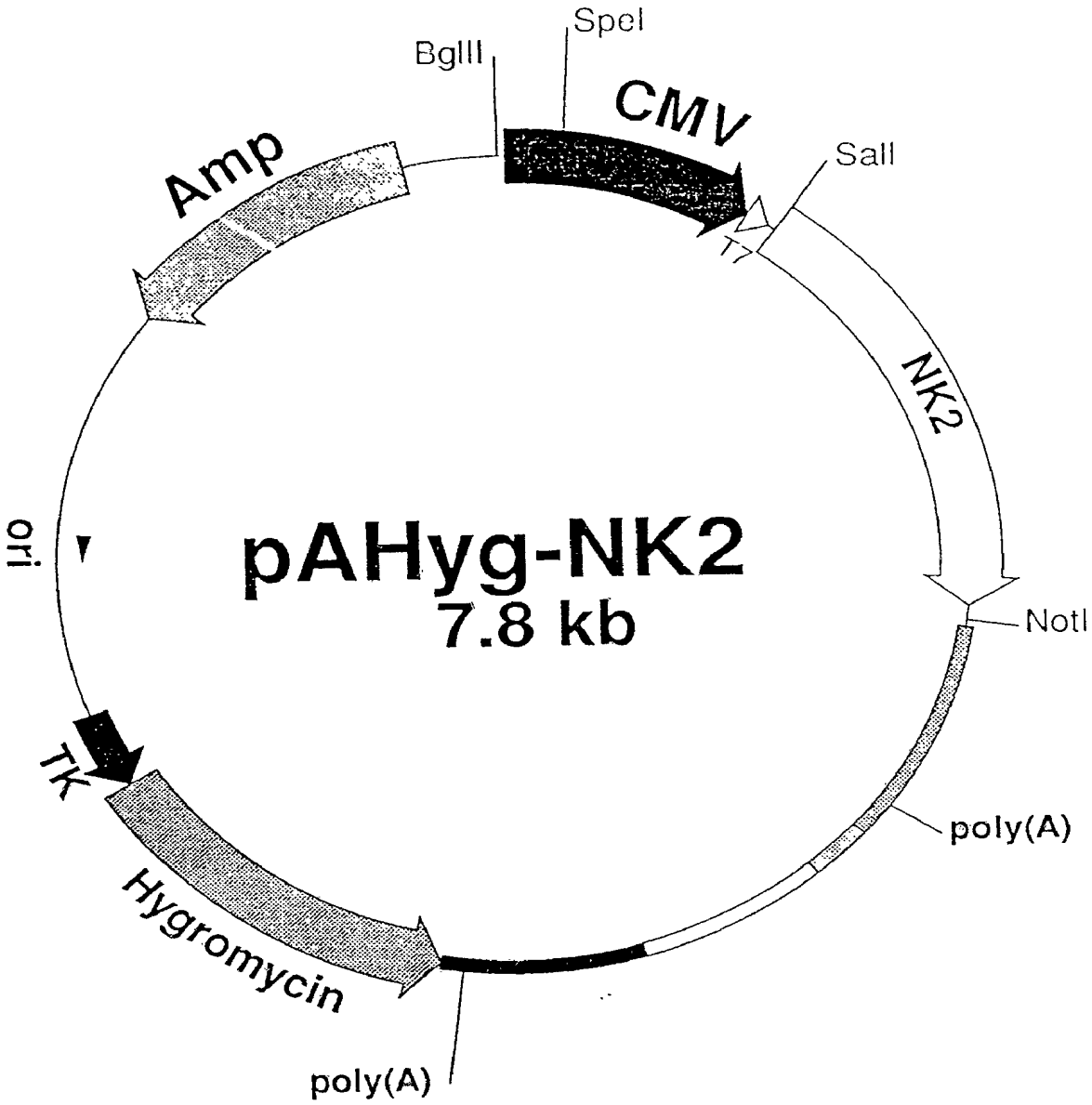

BglII
SpeI
CMV
SalI
Amp
T7
NK2
ori
pAHyg-NK2
7.8 kb
NotI
TK
poly(A)
Hygromycin
poly(A)

Fig. 19B

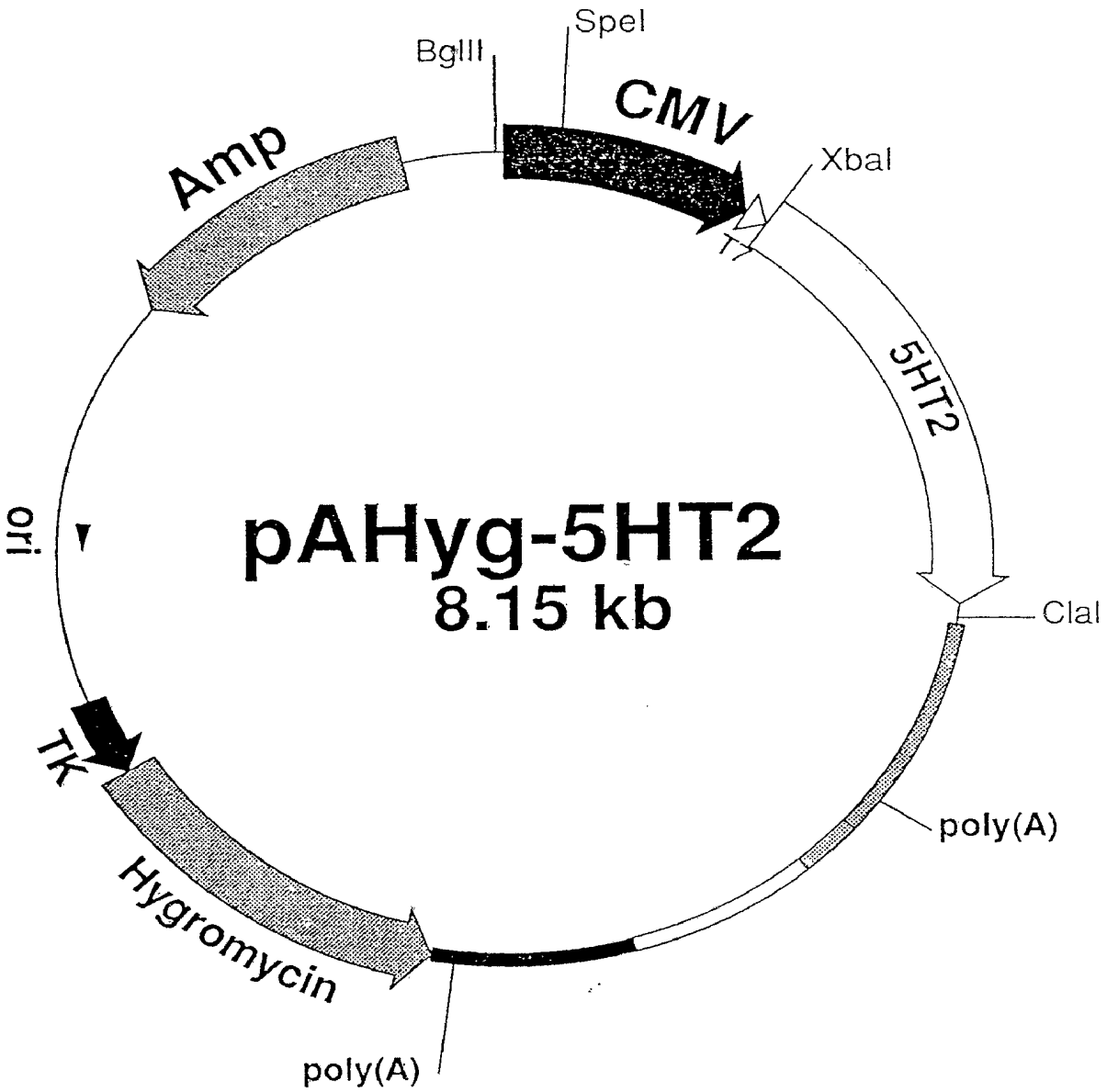

BglII
SpeI
CMV
Amp
XbaI
T7
5HT2
ori
pAHyg-5HT2
8.15 kb
ClaI
TK
poly(A)
Hygromycin
poly(A)

Fig. 20

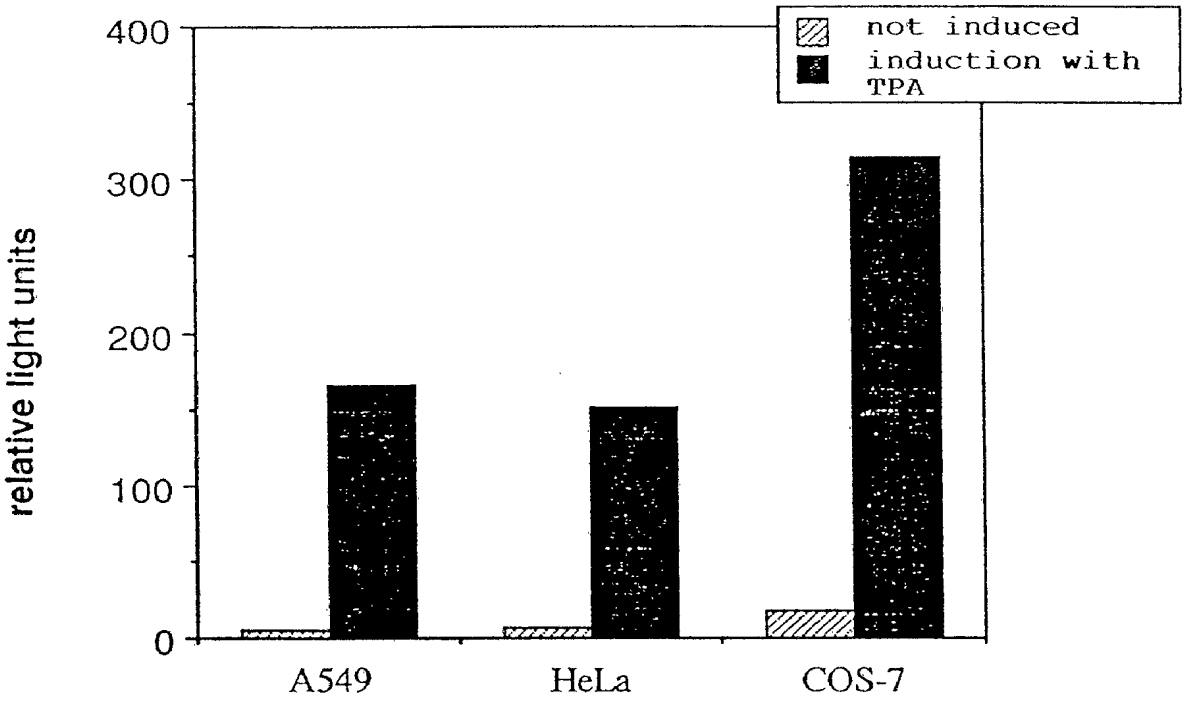
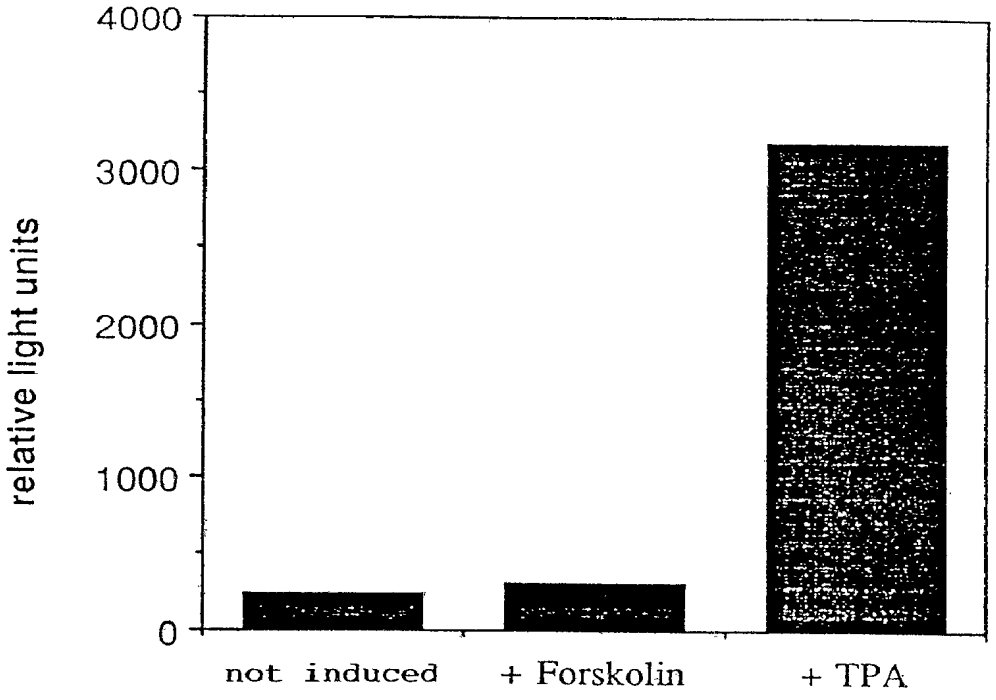
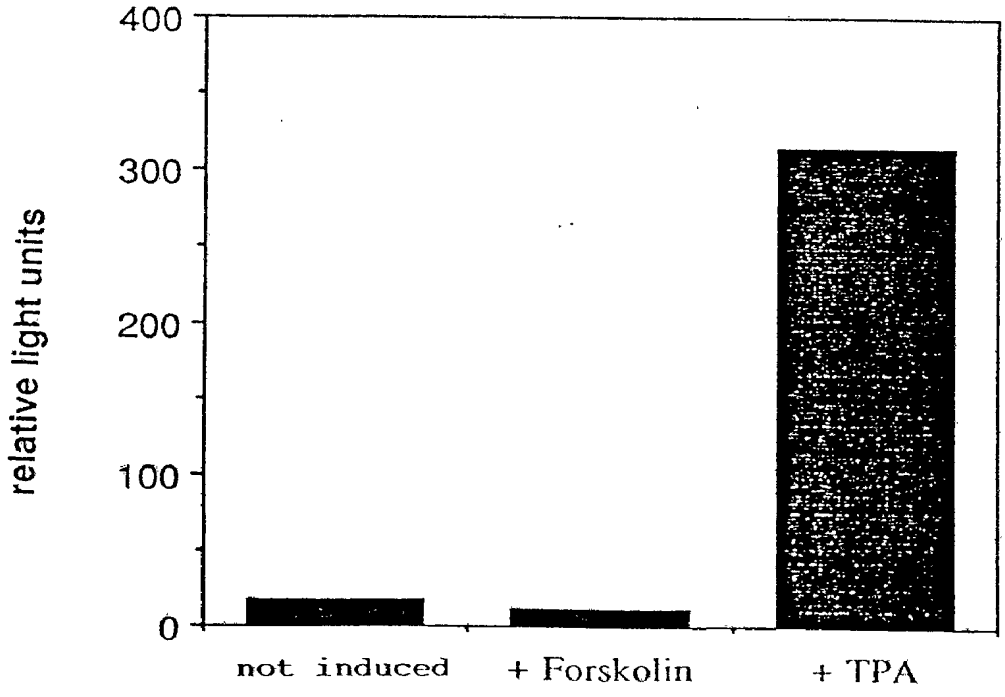

not induced
induction with TPA
relative light units
400
300
200
100
0
A549
HeLa
COS-7

Fig. 21


4000
3000
2000
1000
0
relative light units
not induced
+ Forskolin
+ TPA

A


400
300
200
100
0
relative light units
not induced
+ Forskolin
+ TPA

B

Fig. 22

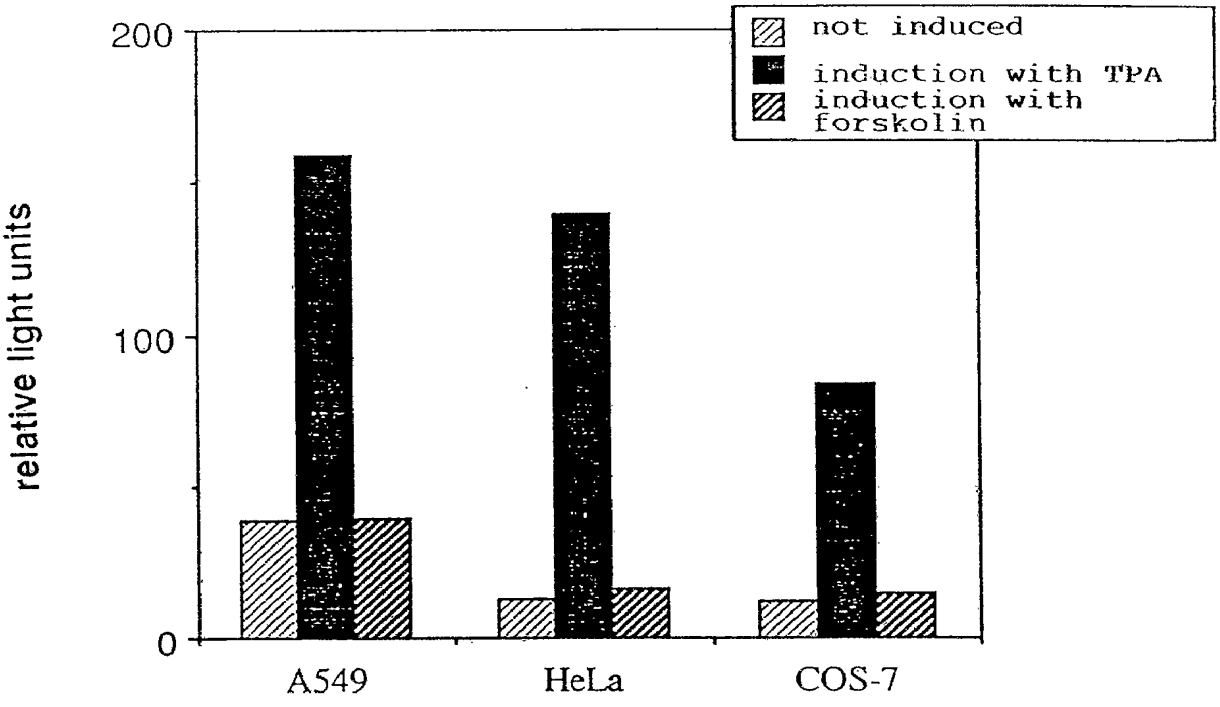

not induced
induction with TPA
induction with forskolin
relative light units
200
100
0
A549
HeLa
COS-7

Fig. 23A

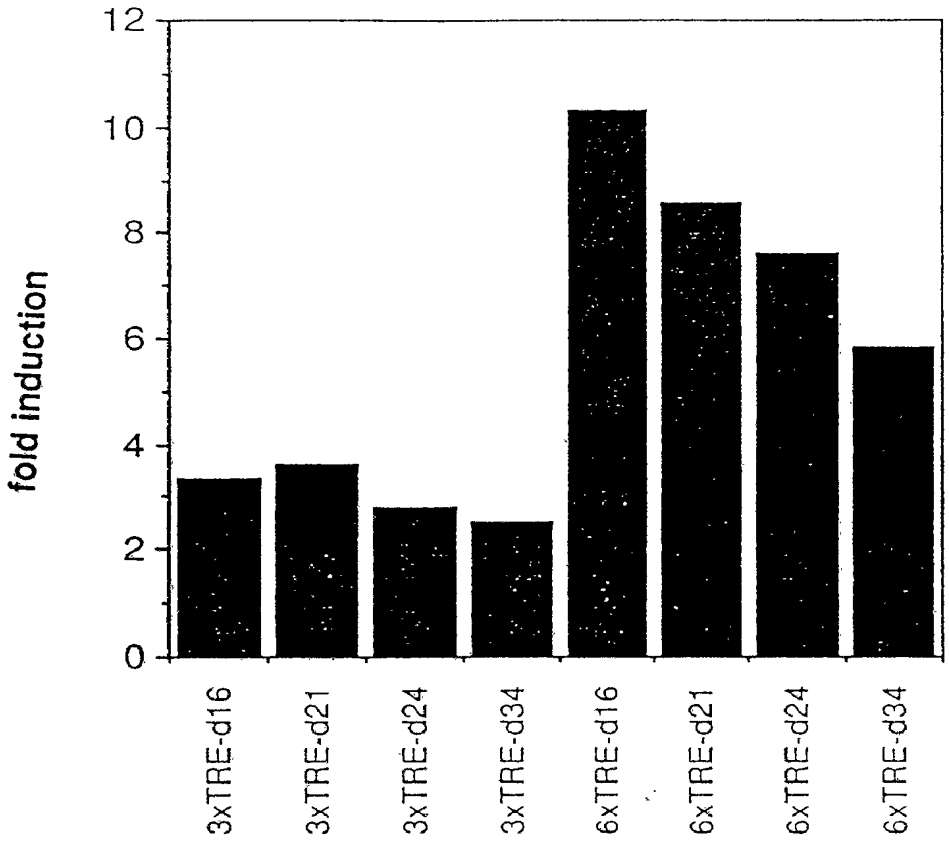

fold induction
0
2
4
6
8
10
12
3xTRE-d16
3xTRE-d21
3xTRE-d24
3xTRE-d34
6xTRE-d16
6xTRE-d21
6xTRE-d24
6xTRE-d34

Fig. 238

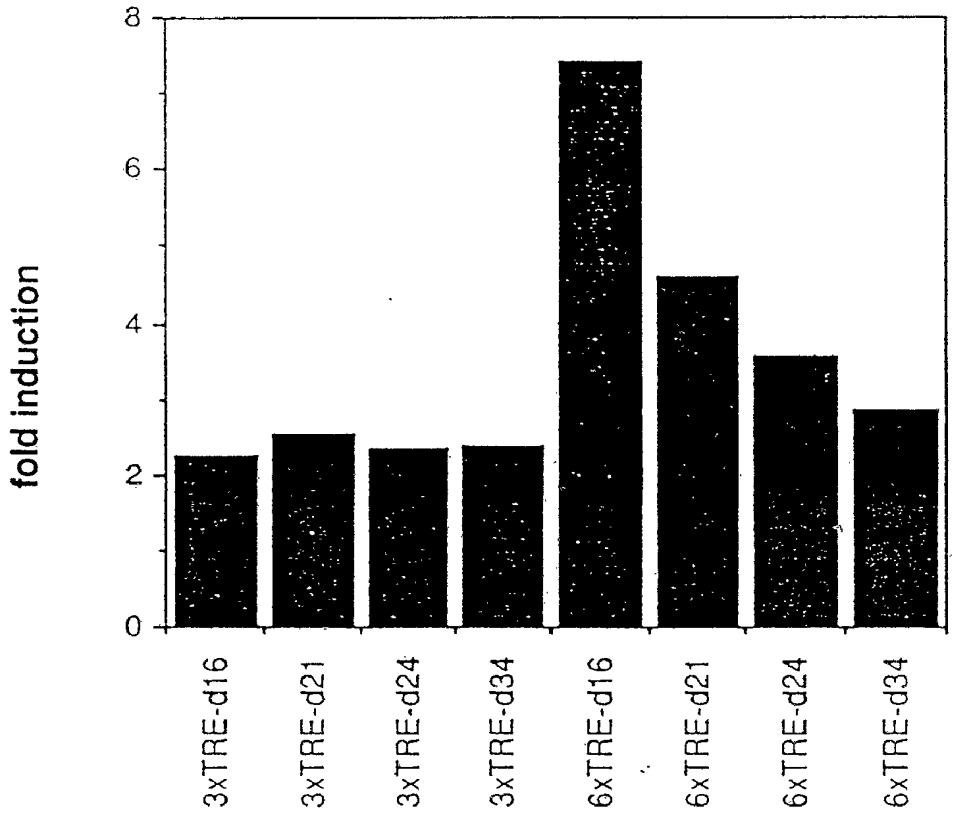

fold induction
0
2
4
6
8
3xTRE-d16
3xTRE-d21
3xTRE-d24
3xTRE-d34
6xTRE-d16
6xTRE-d21
6xTRE-d24
6xTRE-d34

Fig. 24

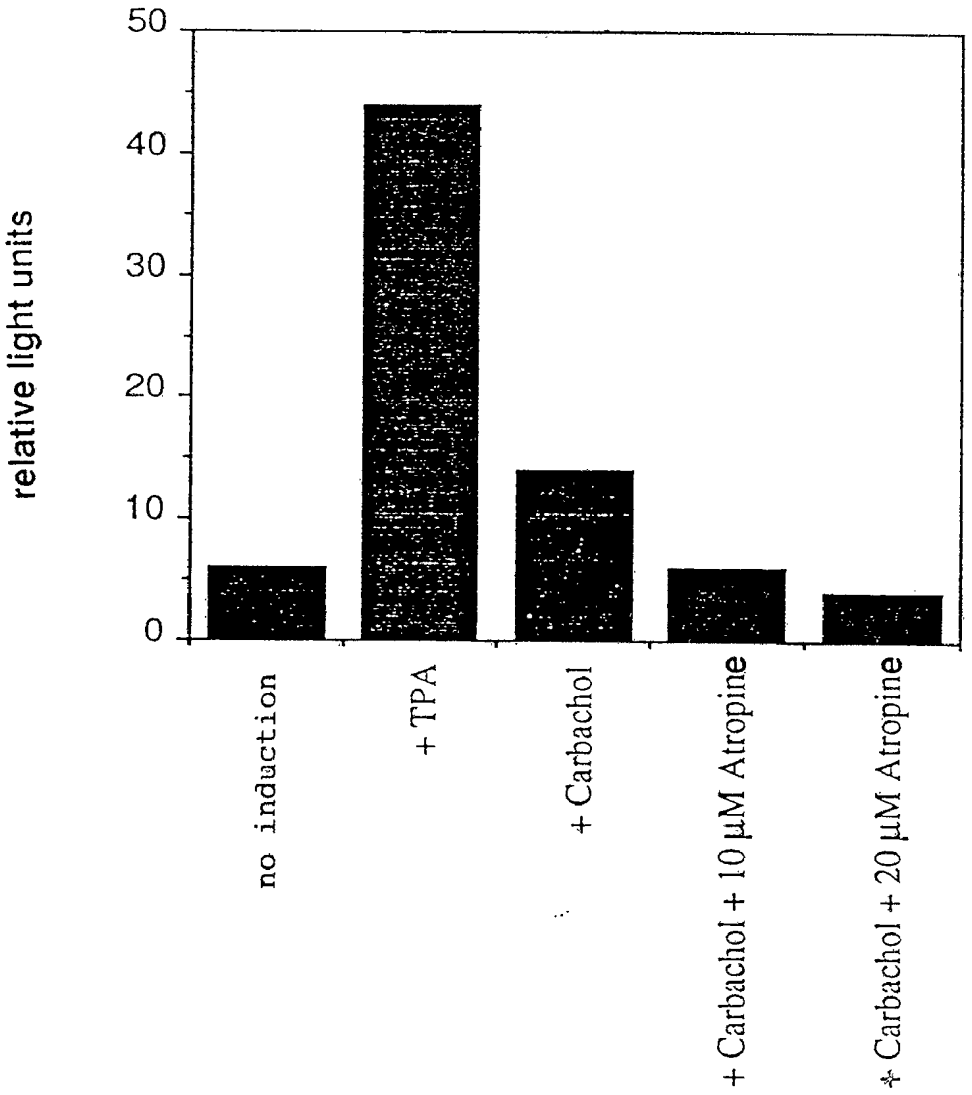

relative light units
50
40
30
20
10
0
no induction
+ TPA
+ Carbachol
+ Carbachol + 10 µM Atropine
+ Carbachol + 20 µM Atropine

Fig. 25

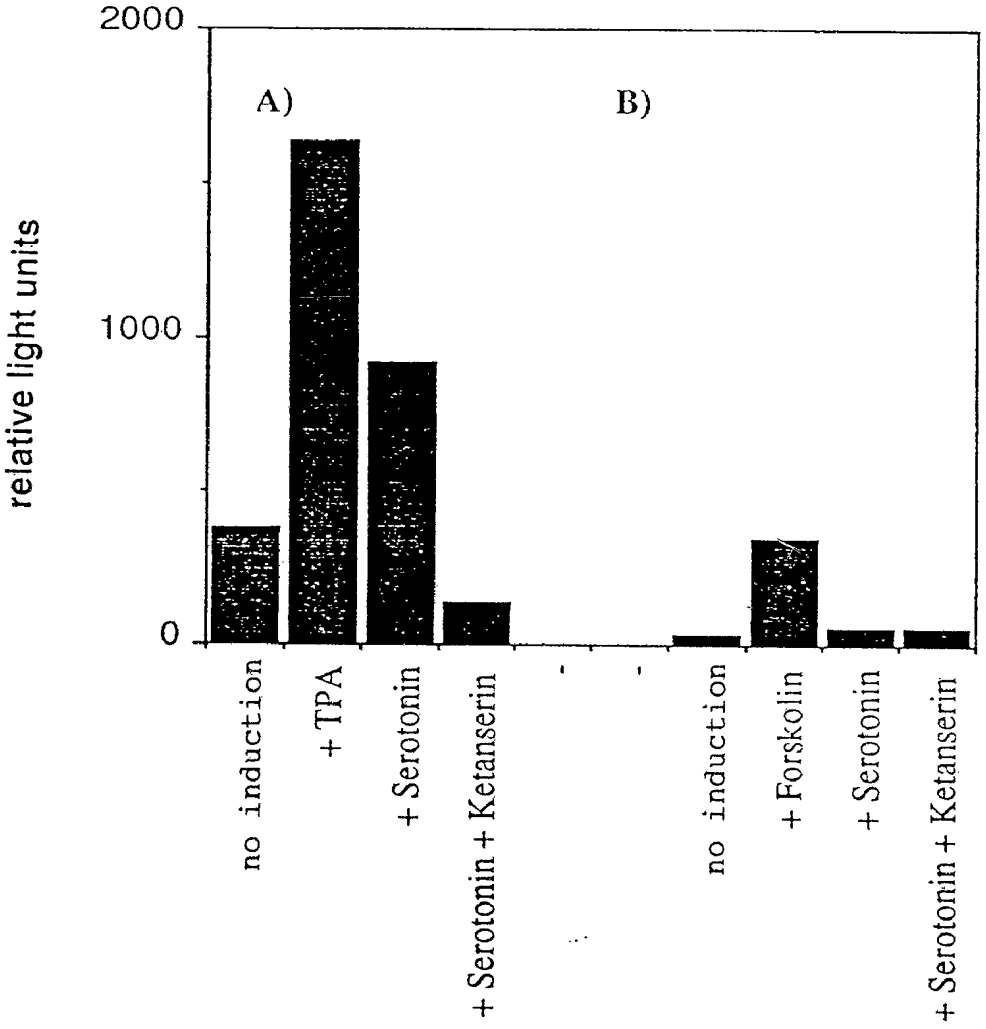

2000
1000
0
relative light units
A)
B)
no induction
+ TPA
+ Serotonin
+ Serotonin + Ketanserin
-
-
no induction
+ Forskolin
+ Serotonin
+ Serotonin + Ketanserin

Fig. 26

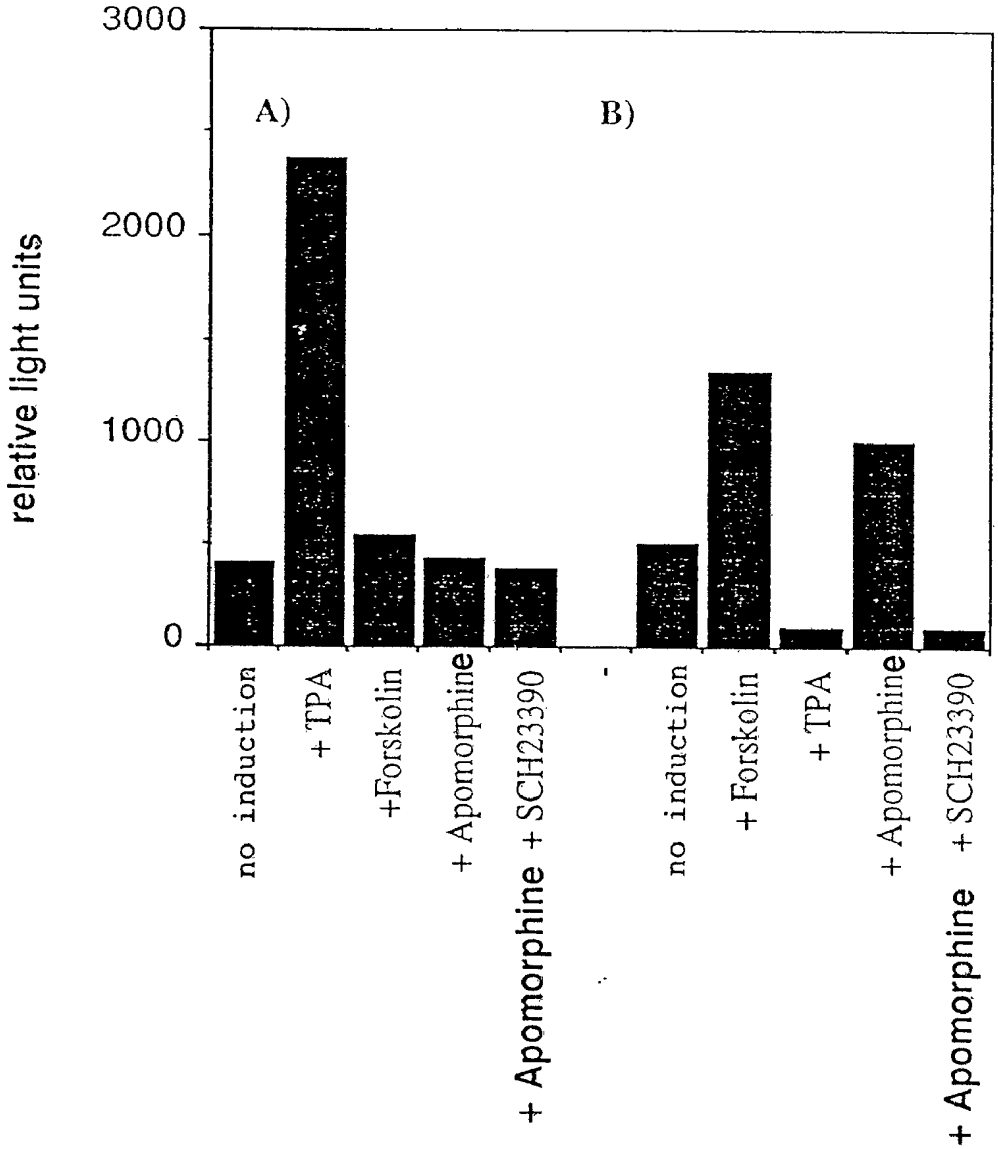

3000
2000
1000
0
relative light units
A)
B)
no induction
+ TPA
+Forskolin
+ Apomorphine
+ Apomorphine + SCH23390
-
no induction
+ Forskolin
+ TPA
+ Apomorphine
+ Apomorphine + SCH23390

Fig. 27

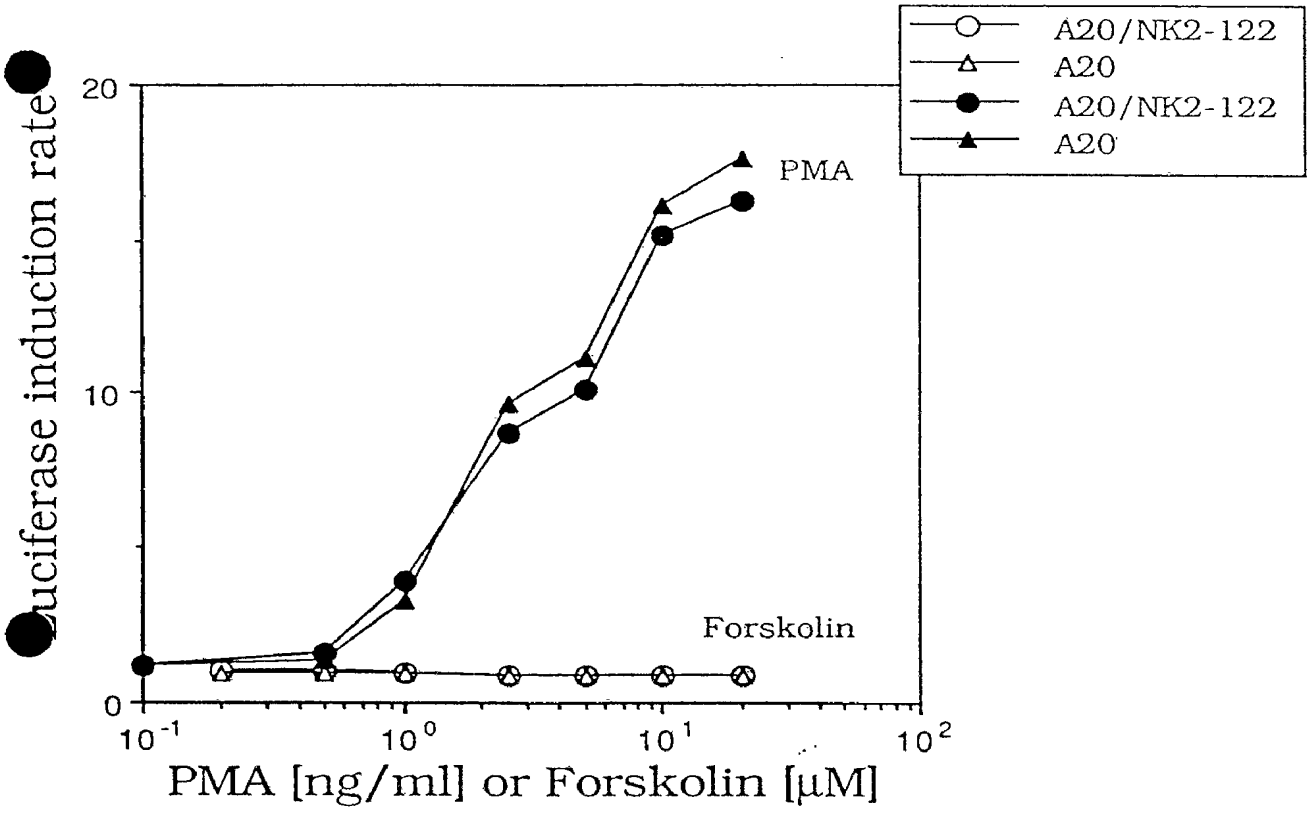

A20/NK2-122
A20
A20/NK2-122
A20
PMA
Forskolin
Luciferase induction rate
20
10
0
10^{-1}
10^{0}
10^{1}
10^{2}
PMA [ng/ml] or Forskolin [μM]

Fig. 28

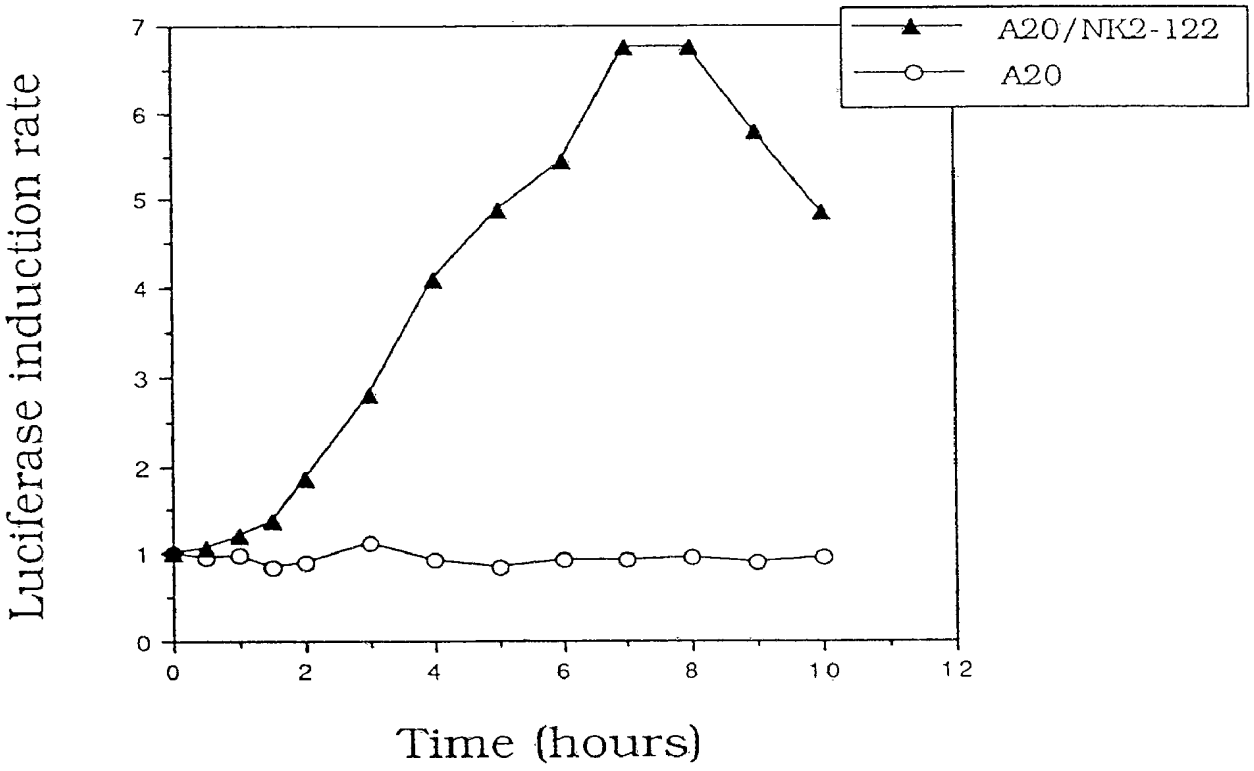

A20/NK2-122
A20
Luciferase induction rate
Time (hours)
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12

Fig. 29A

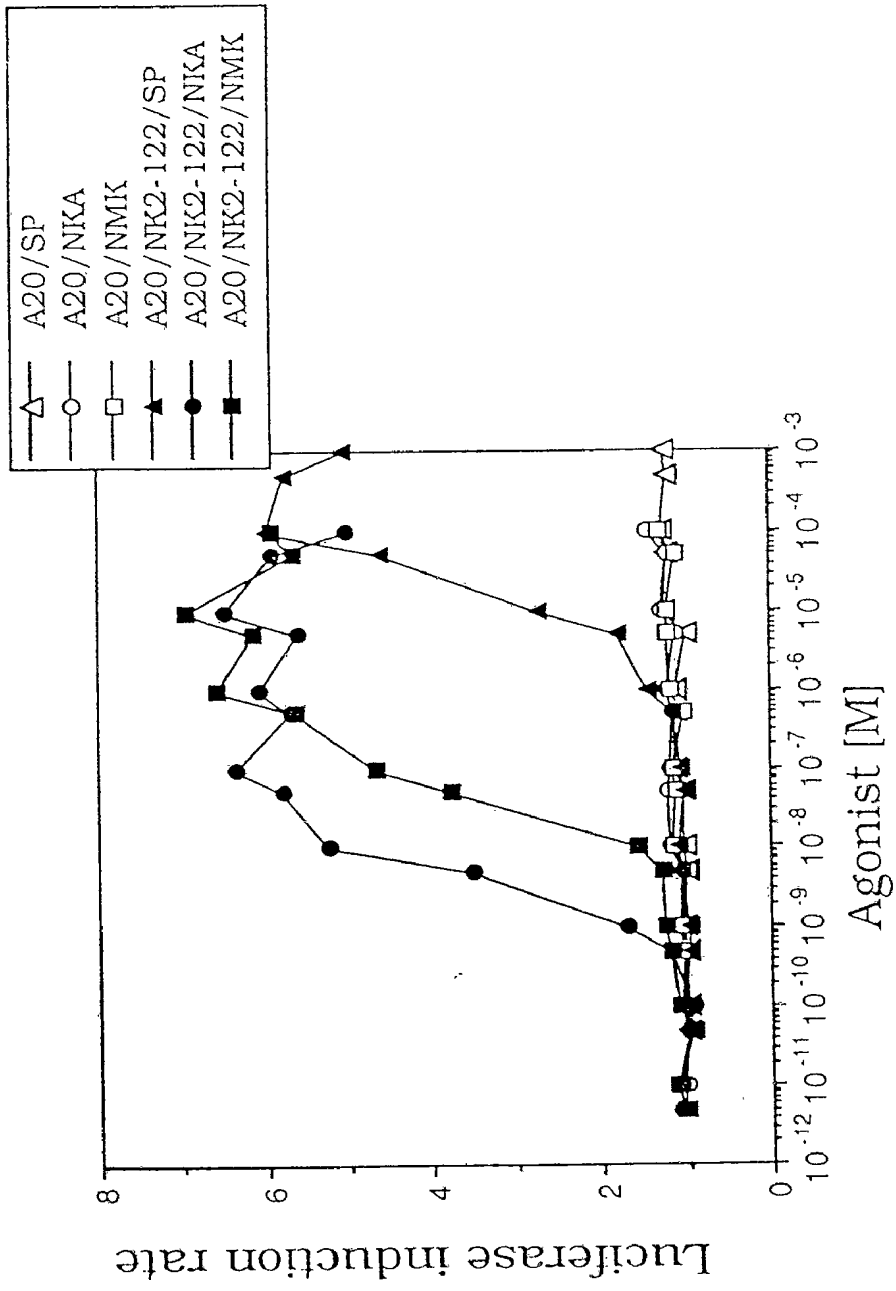

A20/SP
A20/NKA
A20/NMK
A20/NK2-122/SP
A20/NK2-122/NKA
A20/NK2-122/NMK
Luciferase induction rate
0
2
4
6
8
Agonist [M]
10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5 10-4 10-3

Fig. 29B

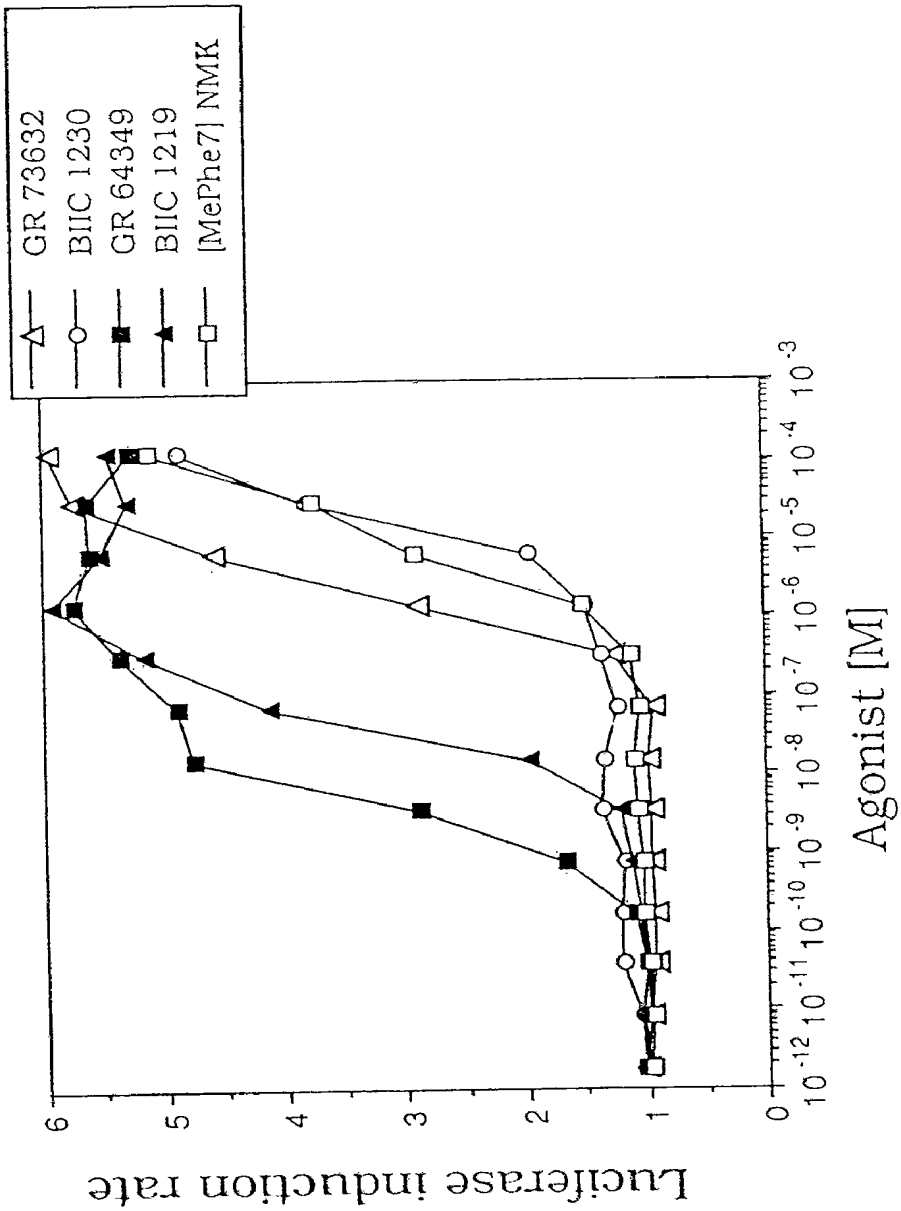

GR 73632
BIIC 1230
GR 64349
BIIC 1219
[MePhe7] NMK
Luciferase induction rate
0
1
2
3
4
5
6
10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
Agonist [M]

Fig. 30

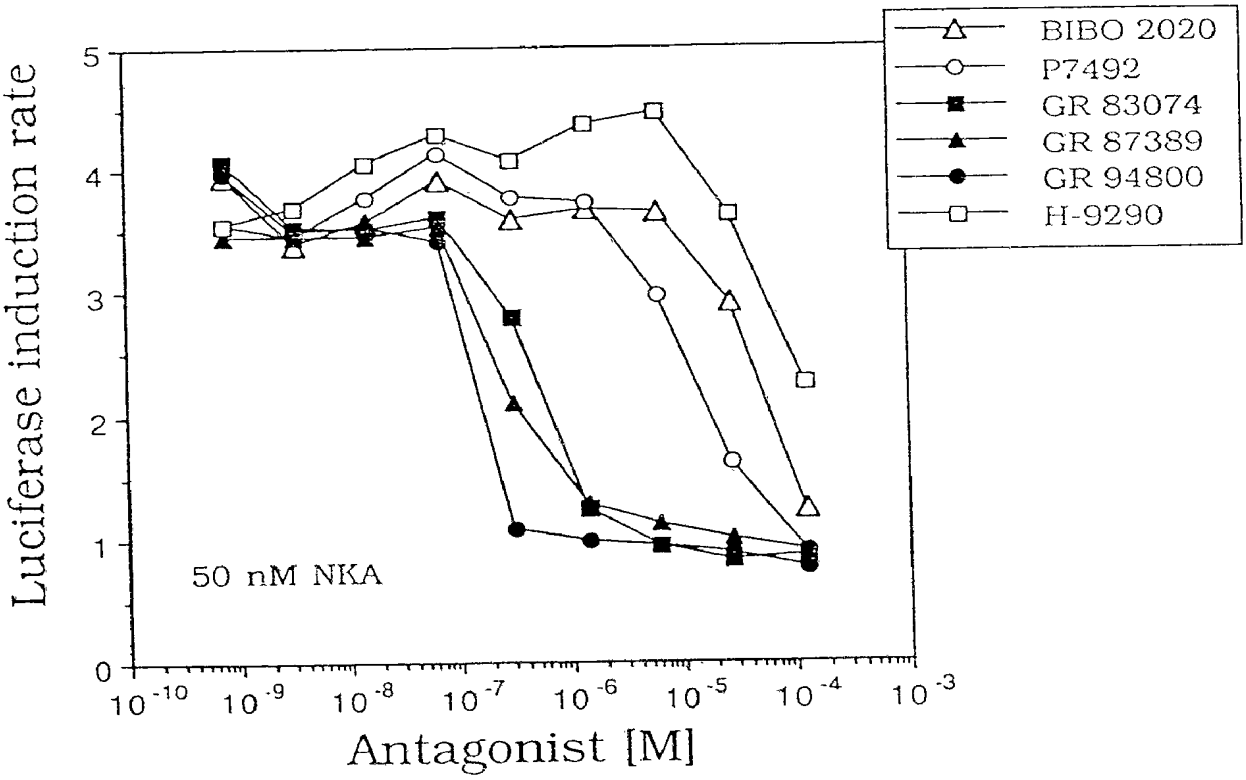

Luciferase induction rate
Antagonist [M]
50 nM NKA
BIBO 2020
P7492
GR 83074
GR 87389
GR 94800
H-9290
0
1
2
3
4
5
10-10
10-9
10-8
10-7
10-6
10-5
10-4
10-3

Fig. 31

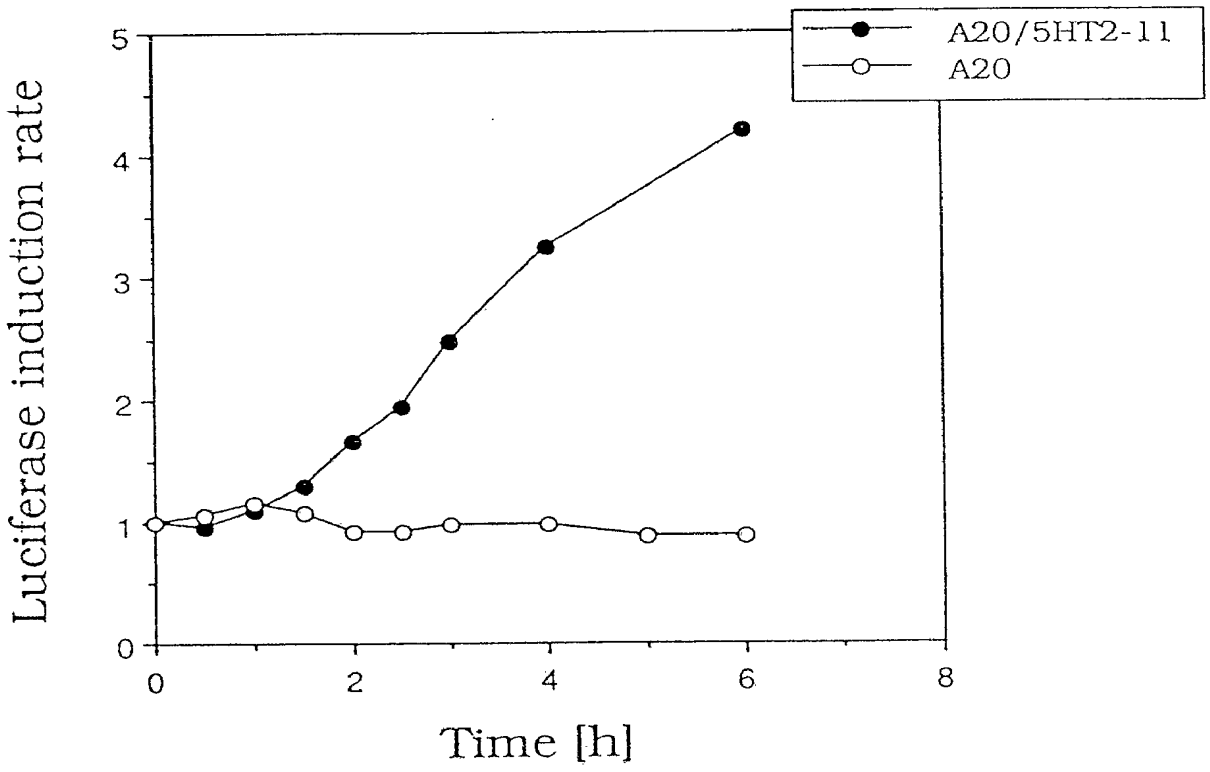

A20/5HT2-11
A20
Luciferase induction rate
Time [h]
0
1
2
3
4
5
0
2
4
6
8

Fig. 32A

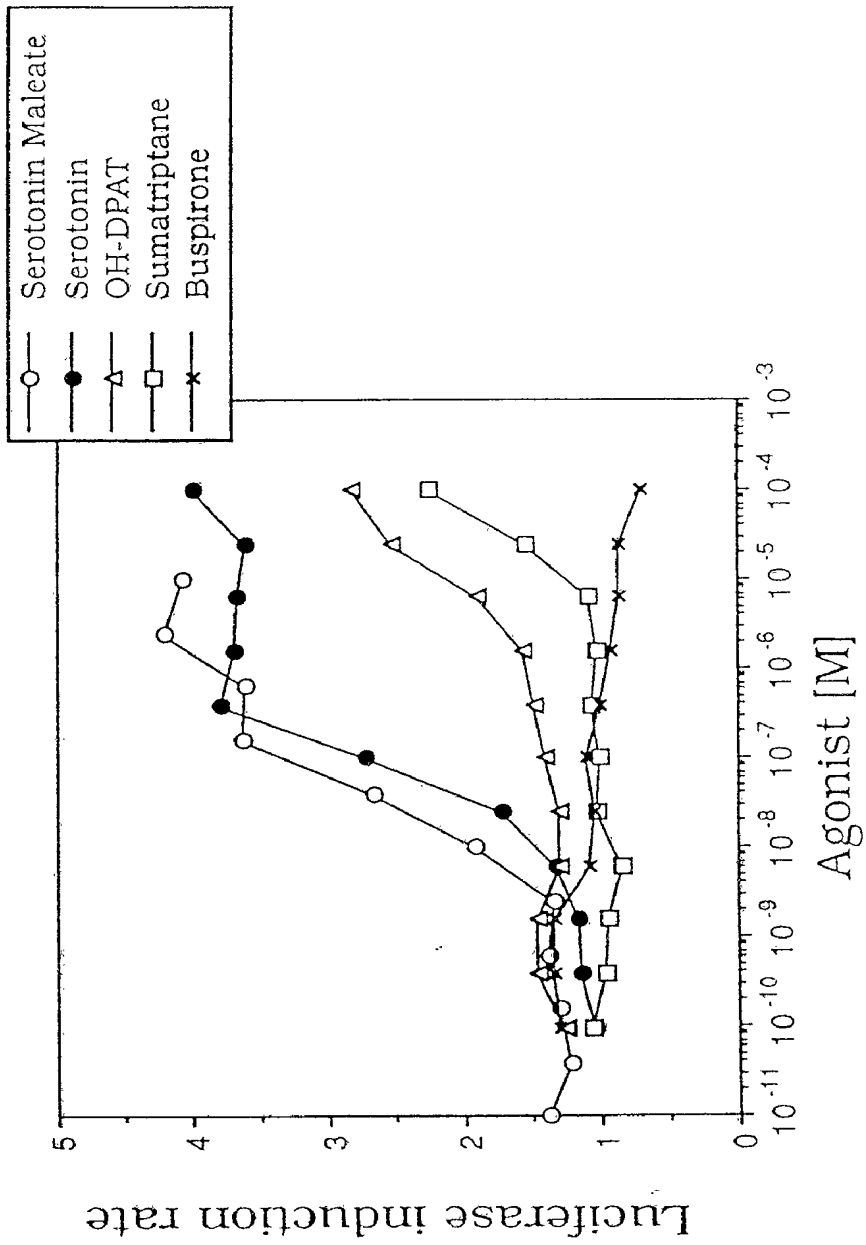

Serotonin Maleate
Serotonin
OH-DPAT
Sumatriptane
Buspirone
Luciferase induction rate
0
1
2
3
4
5
10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
Agonist [M]

Fig. 32B

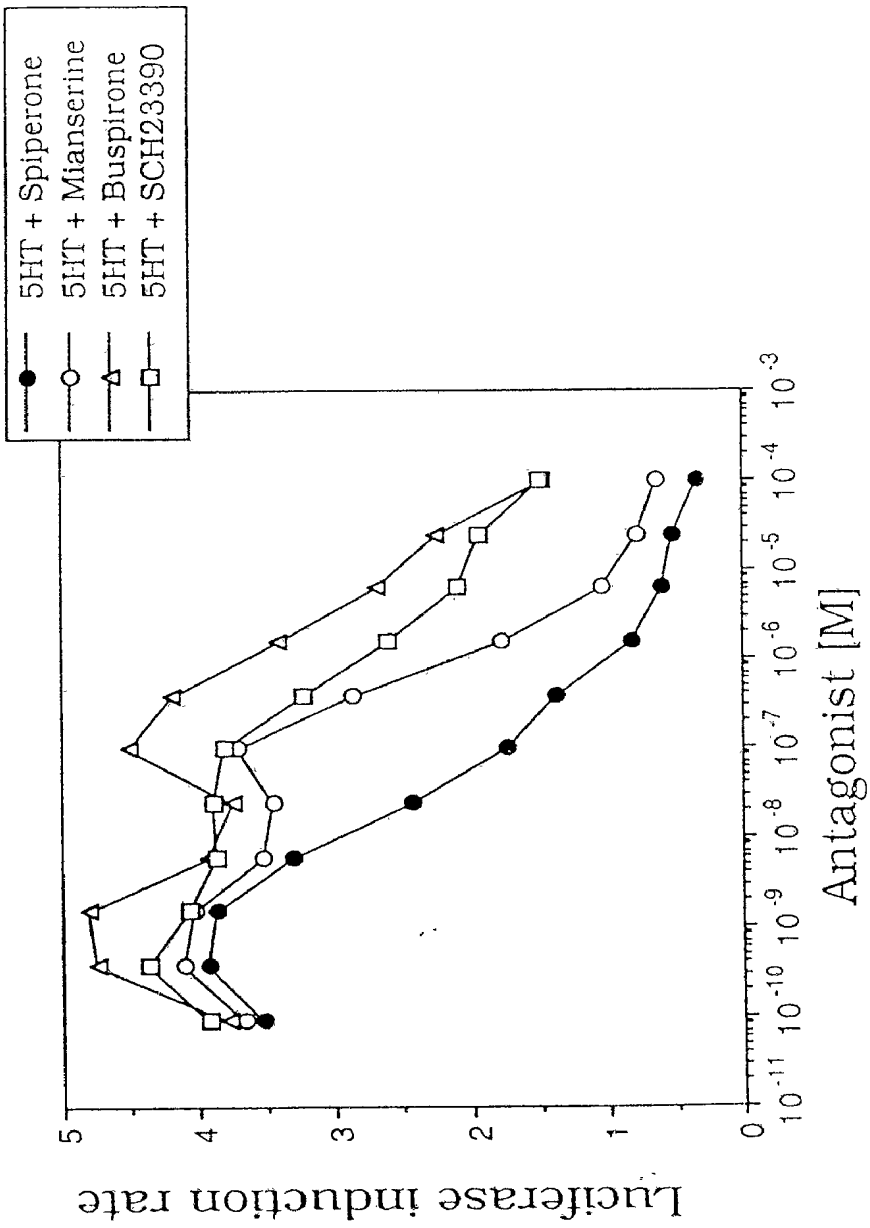

5HT + Spiperone
5HT + Mianserine
5HT + Buspirone
5HT + SCH23390
Luciferase induction rate
0
1
2
3
4
5
10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
Antagonist [M]

Fig. 33

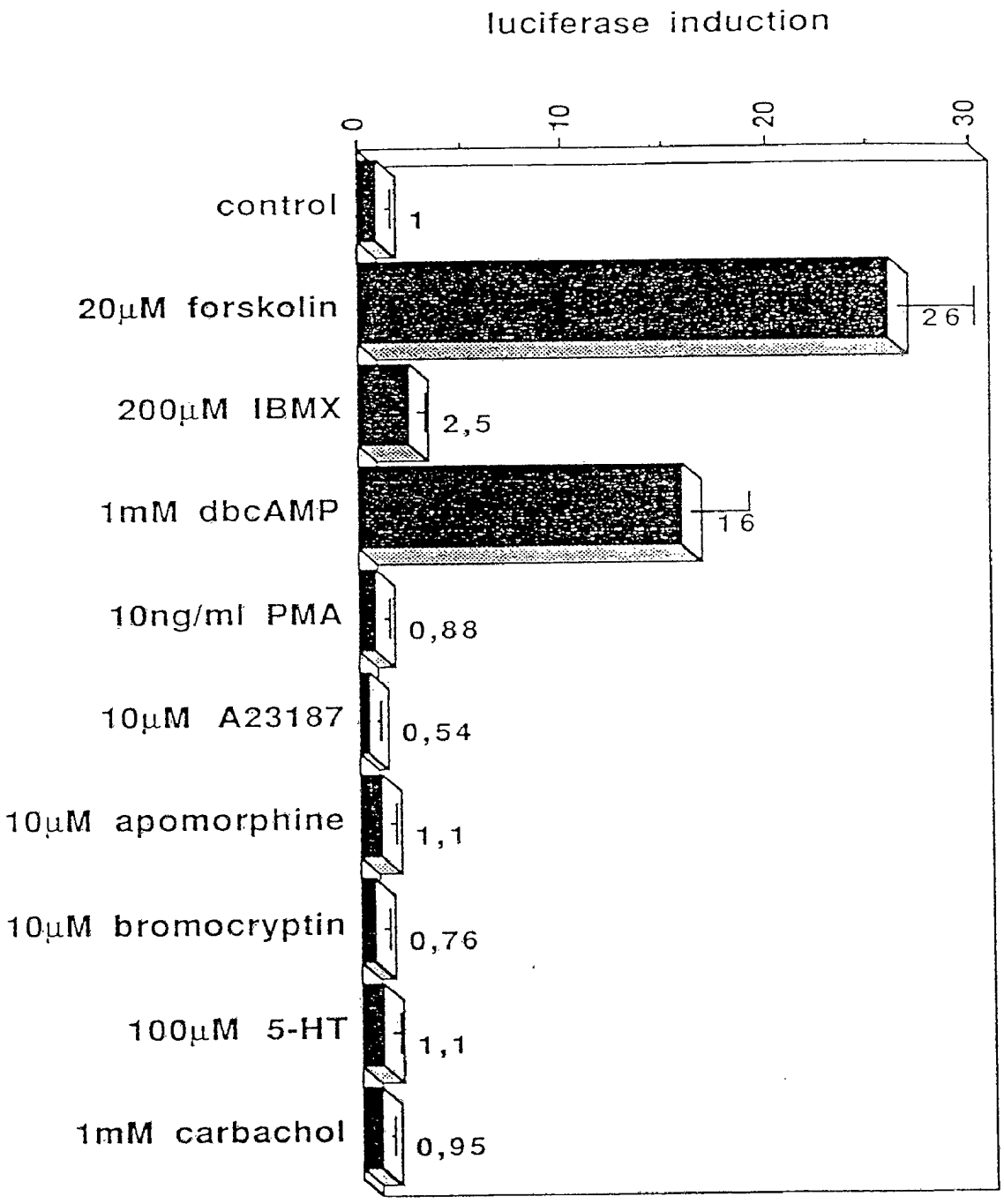

luciferase induction
0
10
20
30
control
1
20µM forskolin
26
200µM IBMX
2,5
1mM dbcAMP
16
10ng/ml PMA
0,88
10µM A23187
0,54
10µM apomorphine
1,1
10µM bromocryptin
0,76
100µM 5-HT
1,1
1mM carbachol
0,95

Fig. 34

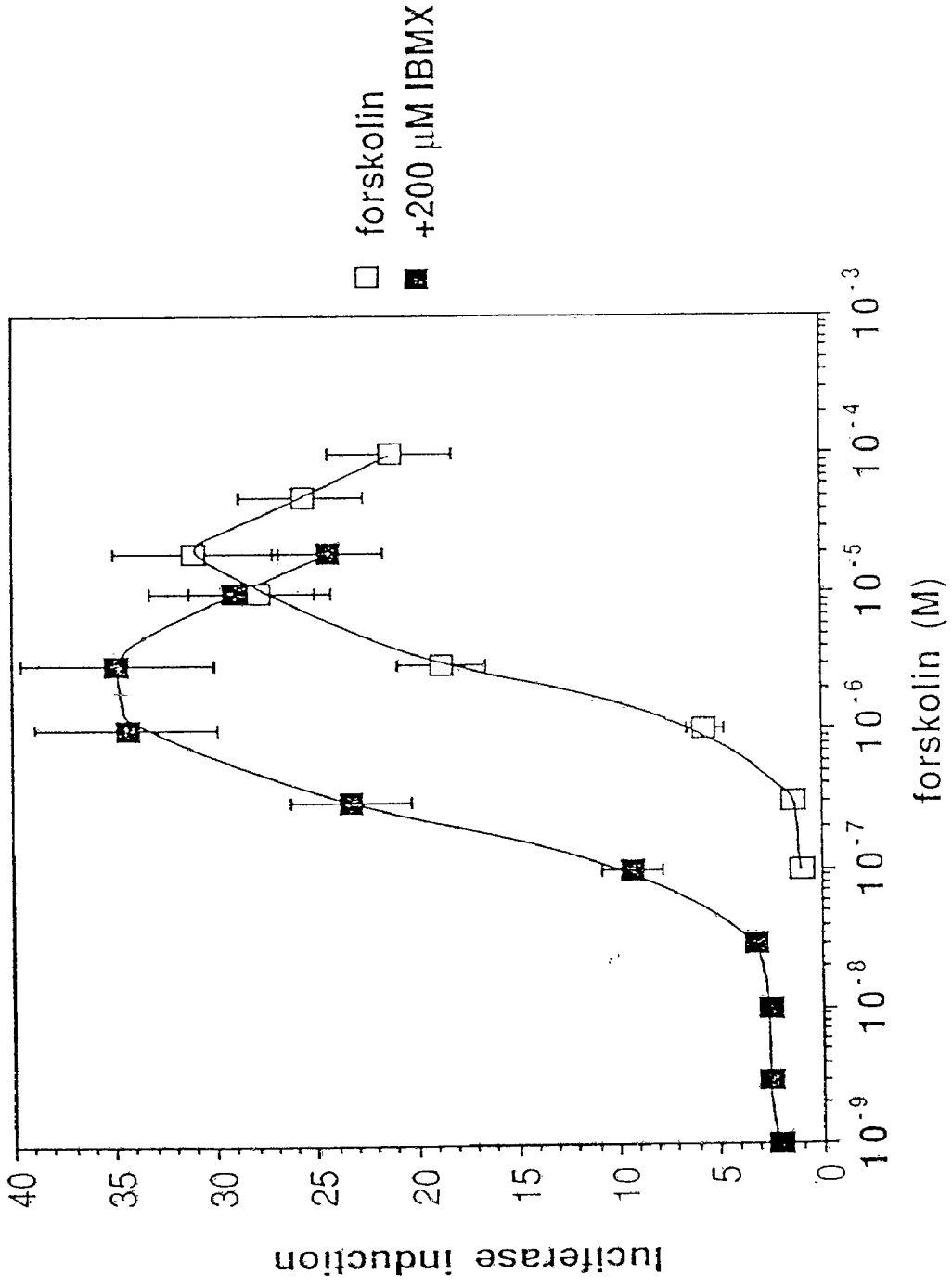

forskolin
+200 µM IBMX
luciferase induction
forskolin (M)
0
5
10
15
20
25
30
35
40
10-9
10-8
10-7
10-6
10-5
10-4
10-3

Fig. 35A

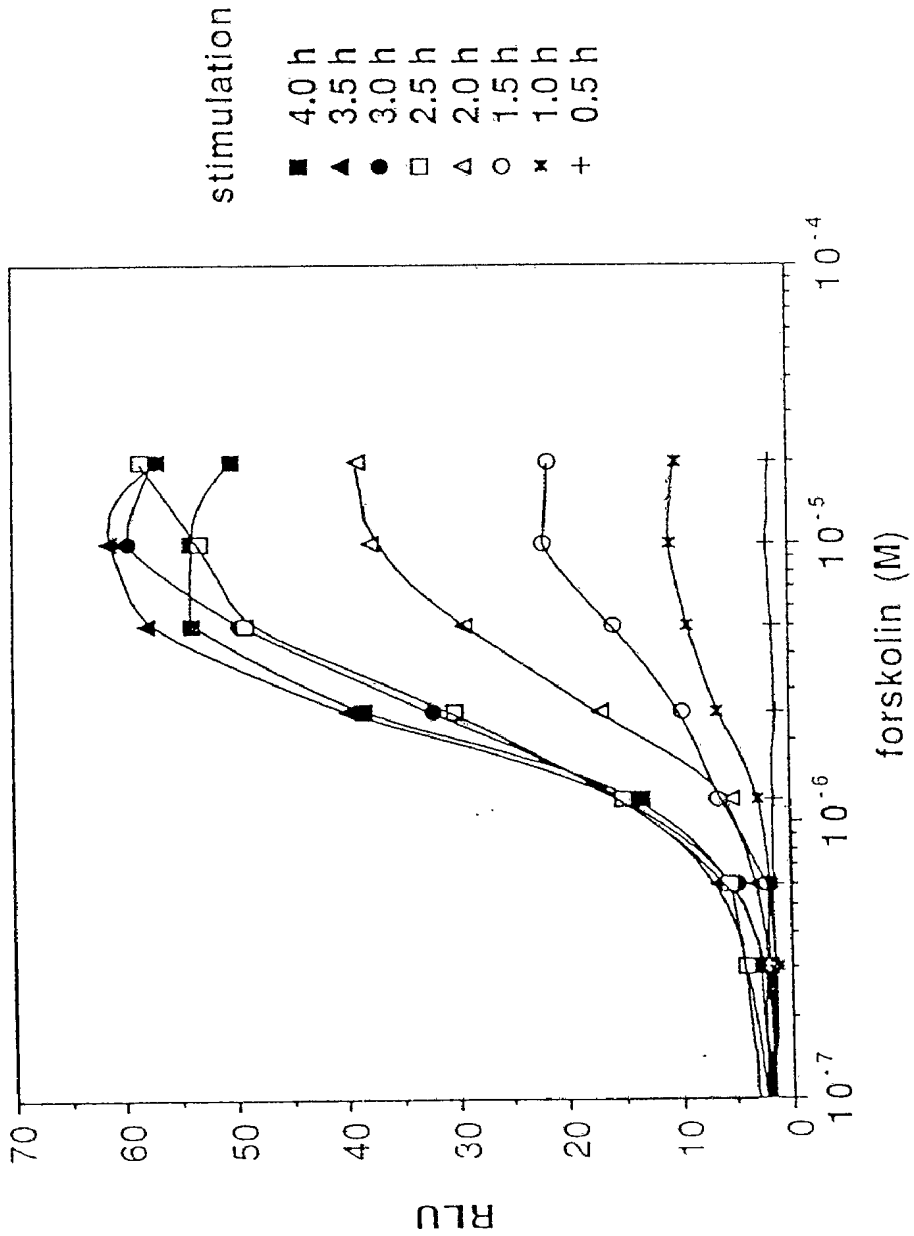

stimulation
4.0 h
3.5 h
3.0 h
2.5 h
2.0 h
1.5 h
1.0 h
0.5 h
RLU
0
10
20
30
40
50
60
70
10^{-7}
10^{-6}
10^{-5}
10^{-4}
forskolin (M)

Fig. 35B

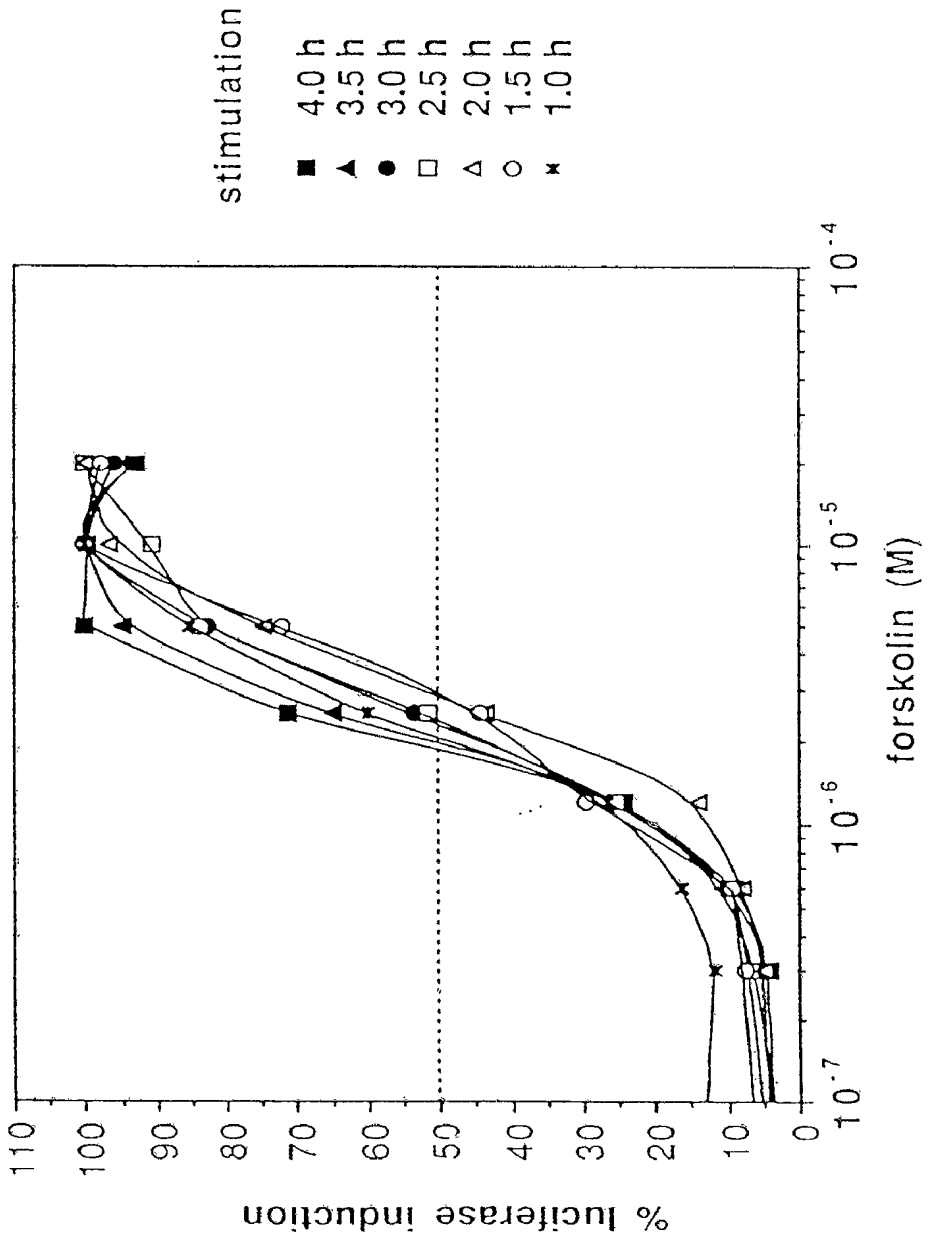

stimulation
4.0 h
3.5 h
3.0 h
2.5 h
2.0 h
1.5 h
1.0 h
% luciferase induction
110
100
90
80
70
60
50
40
30
20
10
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
forskolin (M)

Fig. 36

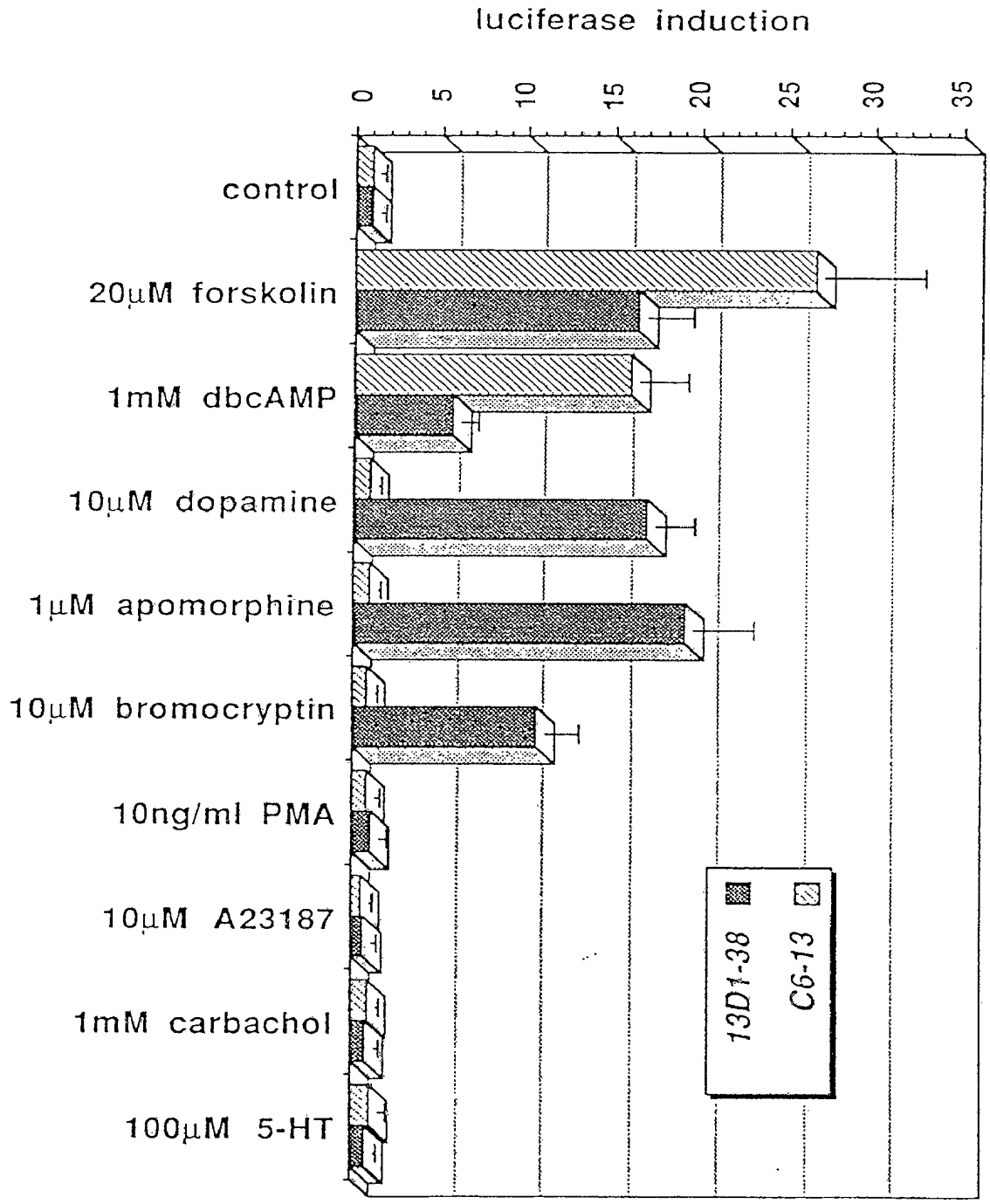

luciferase induction
0
5
10
15
20
25
30
35
control
20μM forskolin
1mM dbcAMP
10μM dopamine
1μM apomorphine
10μM bromocryptin
10ng/ml PMA
10μM A23187
1mM carbachol
100μM 5-HT
13D1-38
C6-13

Fig. 37A

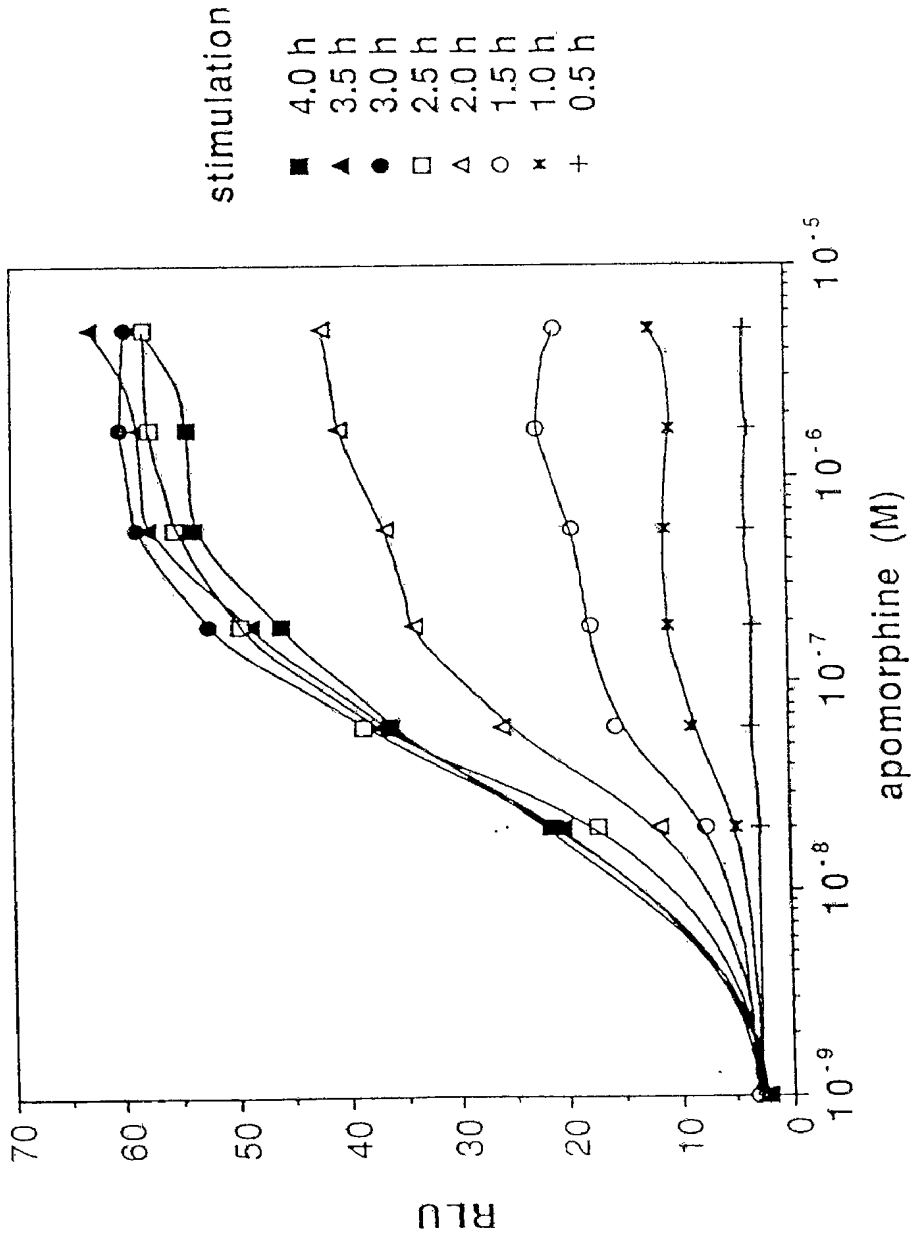

stimulation
4.0 h
3.5 h
3.0 h
2.5 h
2.0 h
1.5 h
1.0 h
0.5 h
RLU
0
10
20
30
40
50
60
70
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
apomorphine (M)

Fig. 37B

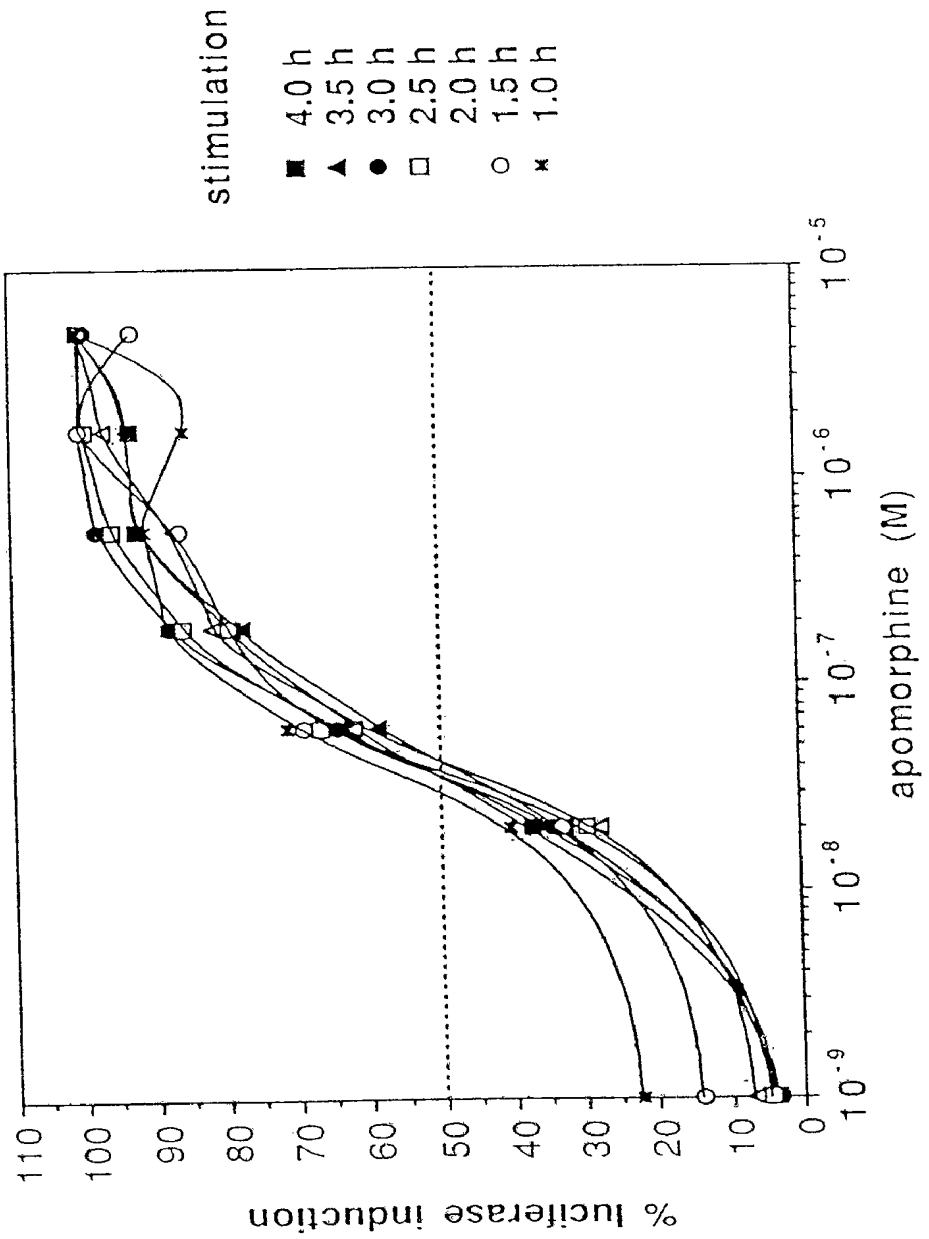

stimulation
4.0 h
3.5 h
3.0 h
2.5 h
2.0 h
1.5 h
1.0 h
% luciferase induction
apomorphine (M)
0
10
20
30
40
50
60
70
80
90
100
110
10^-9
10^-8
10^-7
10^-6
10^-5

Fig. 38A

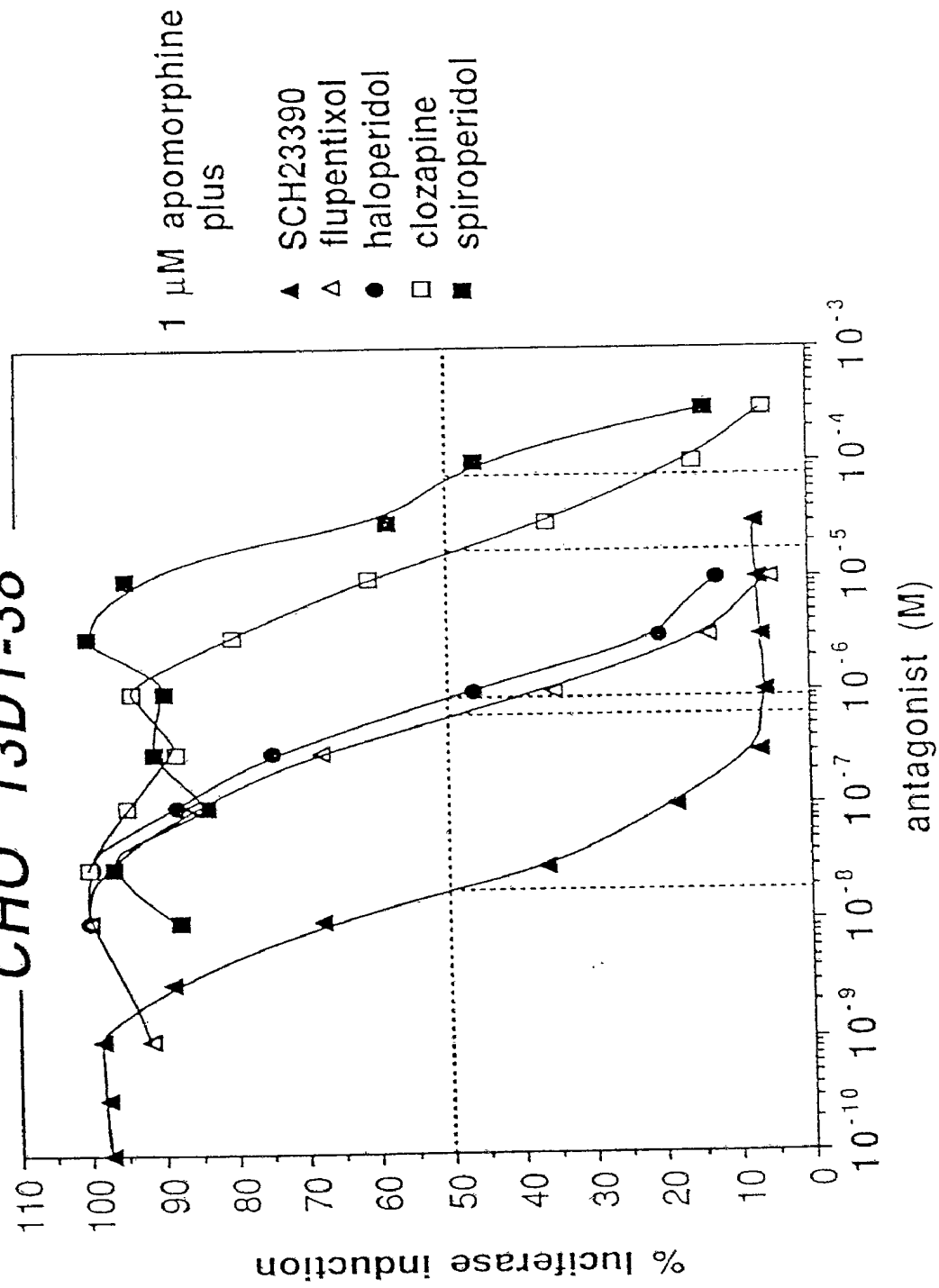

CHO 13D1-38
1 µM apomorphine plus
SCH23390
flupentixol
haloperidol
clozapine
spiroperidol
% luciferase induction
antagonist (M)
110
100
90
80
70
60
50
40
30
20
10
0
10-10
10-9
10-8
10-7
10-6
10-5
10-4
10-3

Fig. 38B

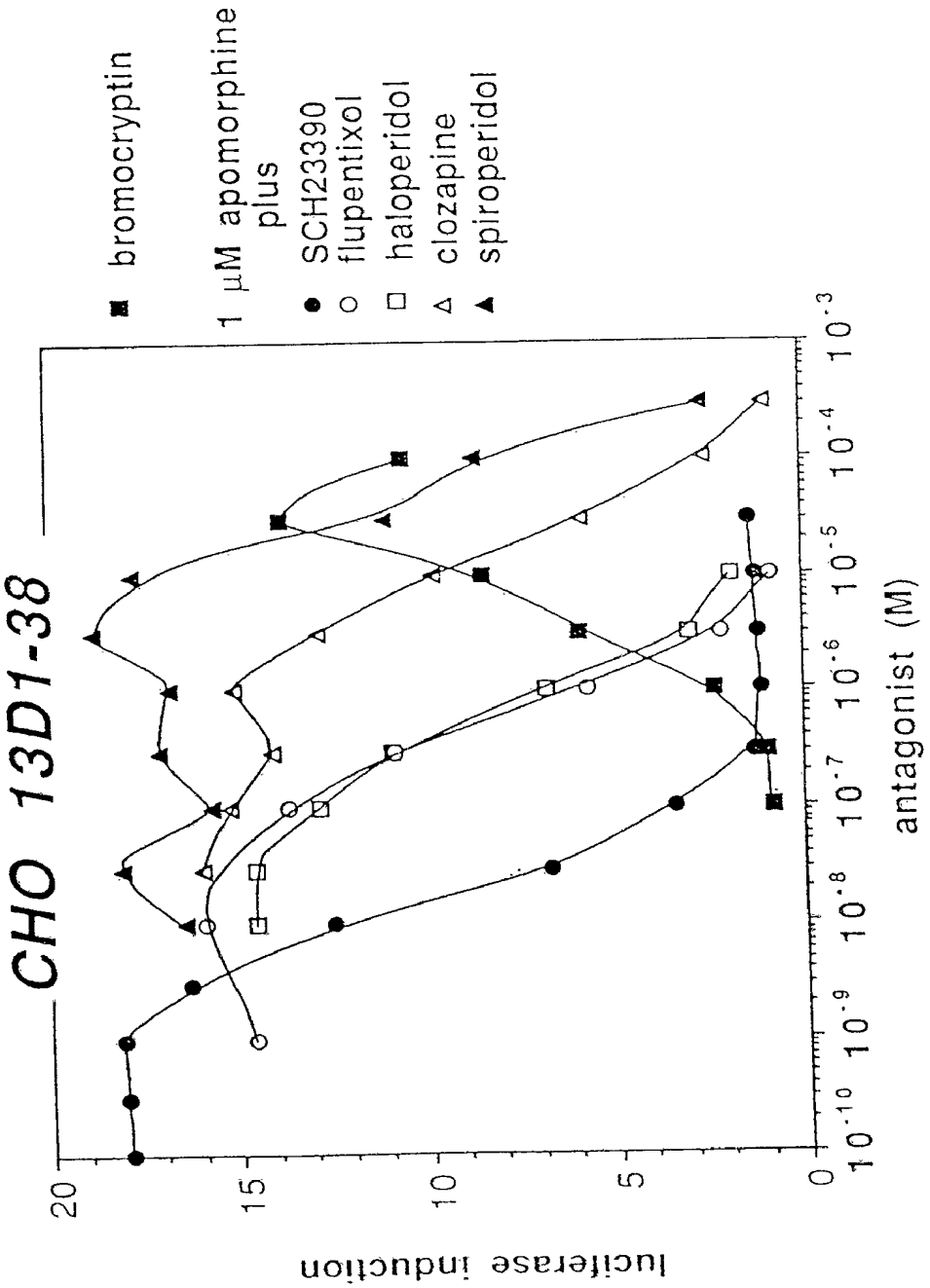

CHO 13D1-38
bromocryptin
1 μM apomorphine plus
SCH23390
flupentixol
haloperidol
clozapine
spiroperidol
luciferase induction
antagonist (M)
0
5
10
15
20
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
10^-3

Fig. 39A

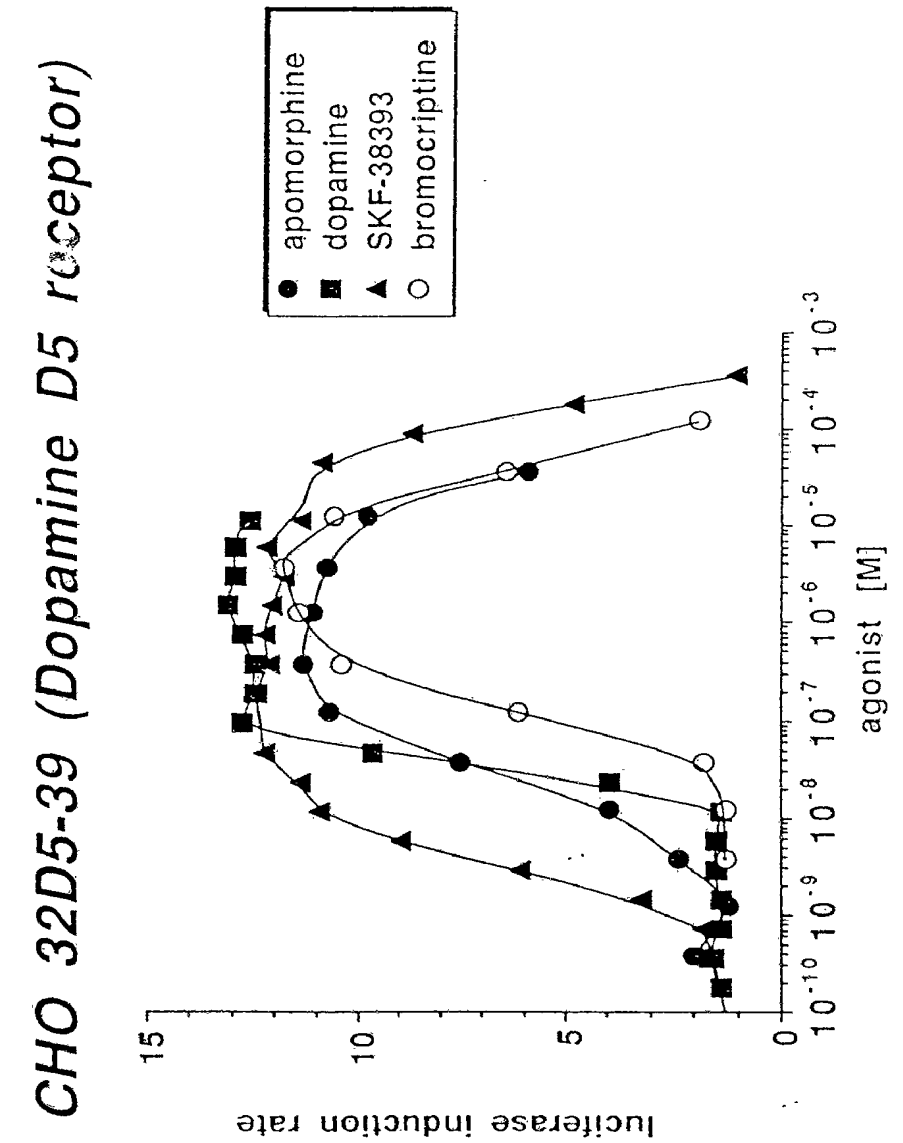

CHO 32D5-39 (Dopamine D5 receptor)
apomorphine
dopamine
SKF-38393
bromocriptine
luciferase induction rate
15
10
5
0
10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
agonist [M]

Fig. 39B

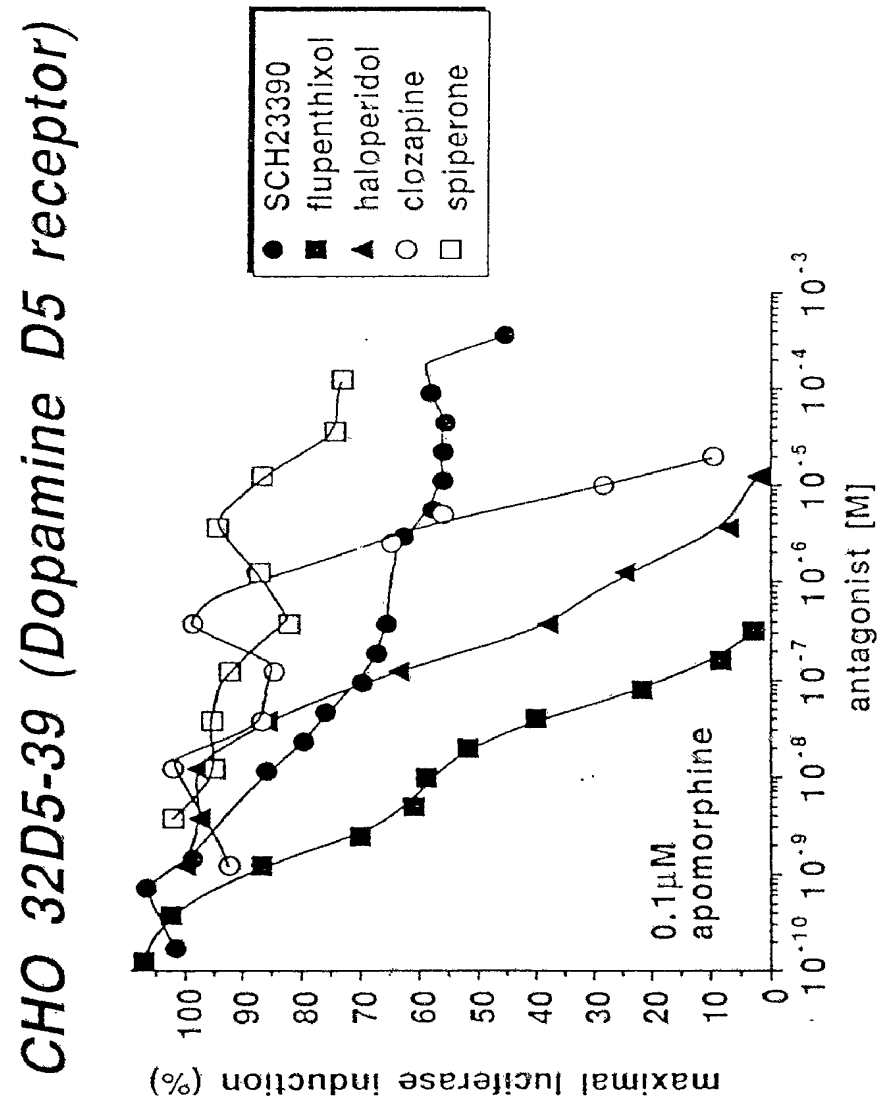
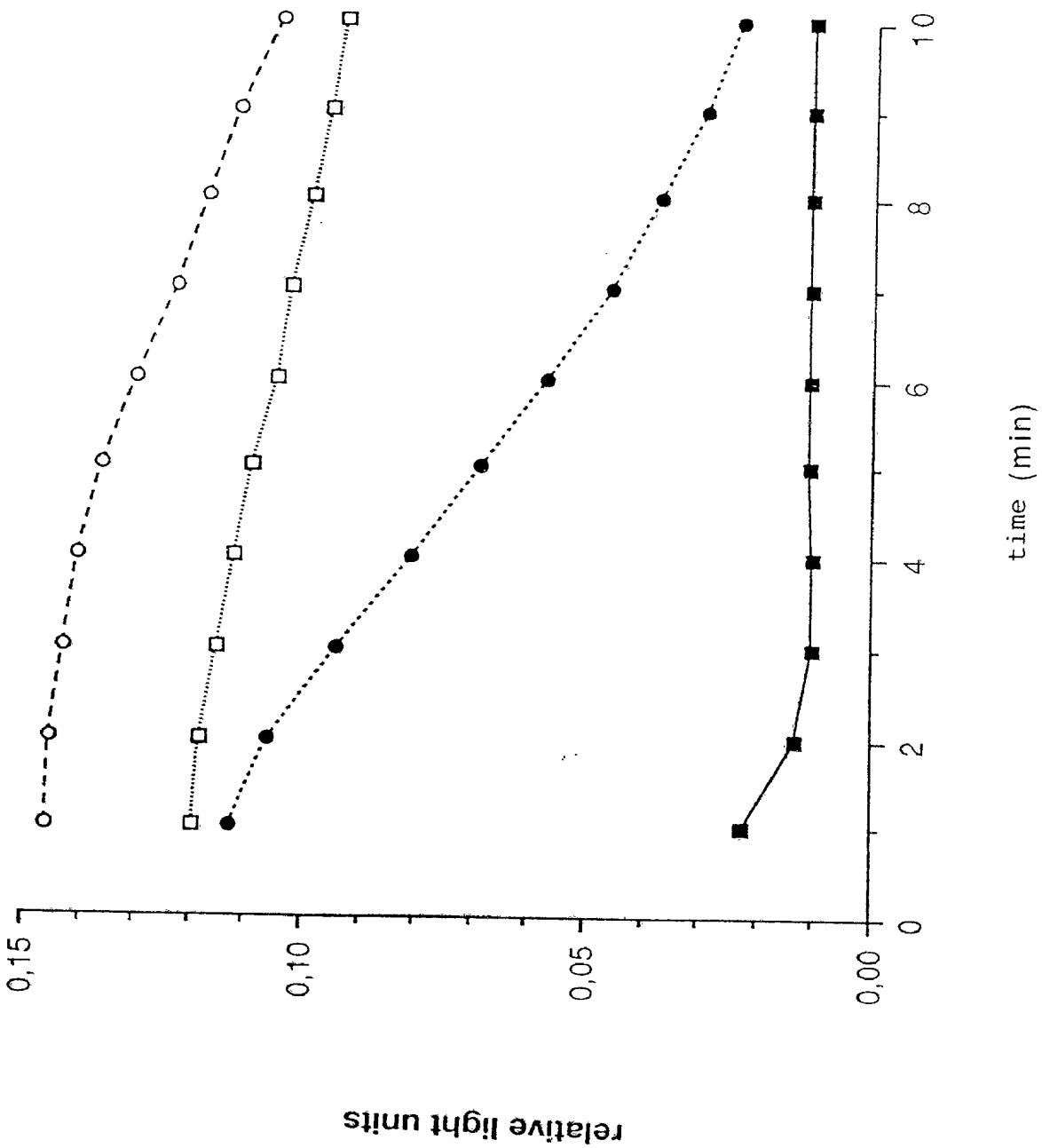

CHO 32D5-39 (Dopamine D5 receptor)
SCH23390
flupenthixol
haloperidol
clozapine
spiperone
0.1µM
apomorphine
maximal luciferase induction (%)
antagonist [M]
0
10
20
30
40
50
60
70
80
90
100
10^-10 10^-9 10^-8 10^-7 10^-6 10^-5 10^-4 10^-3

Fig. 40



INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen PCT/EP 92/02718

I. KLASSIFIKATION DES ANMELDUNGSGEGENSTANDS (bei mehreren Klassifikationssymbolen sind alle anzugeben)[6]

Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC

Int.Cl.5 C 12 Q 1/02 C 12 N 15/12 C 12 N 5/10
C 12 Q 1/66

II. RECHERCHIERTE SACHGEBIETE

Recherchierter Mindestprüfstoff [7]

Klassifikationssytem	Klassifikationssymbole
Int.Cl.5	C 12 Q C 07 K

Recherchierte nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Sachgebiete fallen [8]

III. EINSCHLAGIGE VEROFFENTLICHUNGEN [9]

Art.°	Kennzeichnung der Veröffentlichung [11], soweit erforderlich unter Angabe der maßgeblichen Teile [12]	Betr. Anspruch Nr.[13]
X	WO,A,9115602 (THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES,INC.) 17. Oktober 1991 siehe Seite 5, Zeile 6 - Seite 6, Zeile 27 siehe Seite 29, Zeile 25 - Zeile 33; Ansprüche ---	1
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Bd. 88, Nr. 8, 15. April 1991, WASHINGTON US Seiten 3135 - 3139 MONTMAYEUR J.-P. ET AL. in der Anmeldung erwähnt siehe das ganze Dokument ---	29,30, 35-37
A	MOLECULAR ENDOCRINOLOGY Bd. 5, Nr. 7, Juli 1991, BALTIMORE,USA Seiten 881 - 889 VAN OBBERGHEN-SCHILLING E. ET AL. siehe Zusammenfassung --- -/-	28,38, 40

° Besondere Kategorien von angegebenen Veröffentlichungen [10] :

"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden

"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

IV. BESCHEINIGUNG

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
10-03-1993	18. 05. 93
Internationale Recherchenbehörde	**Unterschrift des bevollmächtigten Bediensteten**
EUROPAISCHES PATENTAMT	E. LUZZATO

III. EINSCHLAGIGE VEROFFENTLICHUNGEN (Fortsetzung von Blatt 2)

Art °	Kennzeichnung der Veröffentlichung, soweit erforderlich unter Angabe der maßgeblichen Teile	Betr. Anspruch Nr.
A	EP,A,0325849 (THE SALK INSTITUTE) 2. August 1989 siehe Seite 8, Zeile 34 - Seite 10, Zeile 33; Ansprüche ---	1,11
A	SCIENCE Bd. 250, 5. Oktober 1990, LANCASTER, PA US Seiten 121 - 123 KING K. ET AL. siehe Zusammenfassung ---	1
A	CELL. Bd. 56, 10. Februar 1989, CAMBRIDGE, NA US Seiten 487 - 493 ASHKENAZI A. ET AL. siehe Zusammenfassung siehe " Discussion " -----	1,38

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP 92/02718

Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 1 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. ☐ Ansprüche Nr.
weil Sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich

2. ☐ Ansprüche Nr.
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich

3. ☐ Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 2 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. Patentansprüche: 1-9 und 11-40
2. Patentansprüche: 10

1. ☐ Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche der internationalen Anmeldung.

2. ☐ Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Internationale Recherchenbehörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

3. ☐ Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Ansprüche der internationalen Anmeldung, für die Gebühren entrichtet worden sind, nämlich auf die Ansprüche Nr.

4. ☒ Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:

1-9 und 11-40

Bemerkungen hinsichtlich eines Widerspruchs

☐ Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.

☒ Die Zahlung zusätzlicher Gebühren erfolgte ohne Widerspruch.

ANHANG ZUM INTERNATIONALEN RECHERCHENBERICHT ÜBER DIE INTERNATIONALE PATENTANMELDUNG NR.

EP 9202718
SA 66870

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben.
Die Angaben über die Familienmitglieder entsprechen dem Stand der Datei des Europäischen Patentamts am 03/05/93
Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie		Datum der Veröffentlichung
WO-A- 9115602	17-10-91	EP-A-	0523187	20-01-93
EP-A- 0325849	02-08-89	US-A-	4981784	01-01-91
		AU-A-	2818889	05-07-89
		JP-T-	3503597	15-08-91
		WO-A-	8905355	15-06-89
		US-A-	5171671	15-12-92

EPO FORM P0473

Für nähere Einzelheiten zu diesem Anhang : siehe Amtsblatt des Europäischen Patentamts, Nr.12/82

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP92/02718

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl^5 : C12Q 1/02 C12N 15/12 C12N 5/10 C12Q 1/66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl^5 : C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 9115602 (THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC.) 17 October 1991 see page 5, line 6 - page 6, line 27 see page 29, line 25 - line 33; claims	1
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Vol. 88, No. 8, 15 April 1991, WASHINGTON US pages 3135 - 3139 MONTMAYEUR J.-P. ET AL. (cited in the application) see the whole document	29,30, 35-37
A	MOLECULAR ENDOCRINOLOGY Vol. 5, No. 7, July 1991, BALTIMORE, USA pages 881 - 889 VAN OBBERGHEN-SCHILLING E. ET AL. see abstract	28,38, 40

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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1993 (10.03.93)	18 May 1993 (18.05.93)
Name and mailing address of the ISA/ European Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP92/02718

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A, 0325849 (THE SALK INSTITUTE) 2 August 1989 see page 8, line 34 - page 10, line 33; claims	1,11
A	SCIENCE Vol. 250, 5 October 1990, LANCASTER, PA US pages 121 - 123 KING K. ET AL. see abstract	1
A	CELL. Vol. 56, 10 February 1989, CAMBRIDGE, NA US pages 487 - 493 ASHKENAZI A. ET AL. see abstract see "Discussion"	1,38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP92/02718

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1 - Claims 1-9 and 11-40
2 - Claim 10

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-9 and 11-40

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

ANHANG ZUM INTERNATIONALEN RECHERCHENBERICHT ÜBER DIE INTERNATIONALE PATENTANMELDUNG NR.

EP 9202718
SA 66870

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben.
Die Angaben über die Familienmitglieder entsprechen dem Stand der Datei des Europäischen Patentamts am 03/05/93
Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie		Datum der Veröffentlichung
WO-A- 9115602	17-10-91	EP-A-	0523187	20-01-93
EP-A- 0325849	02-08-89	US-A-	4981784	01-01-91
		AU-A-	2818889	05-07-89
		JP-T-	3503597	15-08-91
		WO-A-	8905355	15-06-89
		US-A-	5171671	15-12-92

EPO FORM P0473

Für nähere Einzelheiten zu diesem Anhang : siehe Amtsblatt des Europäischen Patentamts, Nr.12/82